Contribution of Human Papillomavirus E6 PDZ-Binding Activity to Virus-Induced Pathogenesis

Thesis

Contribution of Human Papillomavirus E6 PDZ-binding Activity to Virus-induced Pathogenesis

Christian Kranjec

This thesis is submitted for the degree Doctor of Philosophy in the faculty of Life Sciences of the Open University, UK

International Centre for Genetic Engineering and Biotechnology (ICGEB), Trieste-Italy

Director of Studies: Lawrence Banks, PhD

Second Supervisor: Sally Roberts, PhD

September, 2013

DATE of SUBMISSION: 17 SEPTEMBER 2013

DATE OF AWARD: 6 DECEMBER 2013

Acknowledgments

I have to thank many people for constant encouragement and support that made the preparation of this thesis possible.

My mother and my whole family whose presence encouragement and support (not only moral) made the preparation of this thesis possible. To my father who, although no longer with me, gave me the strength to reach this point.

My mentor, Lawrence Banks, for his constant supervision, critical analysis of the data and precious advices and suggestions. Miranda Thomas for the patient correction of my imperfect English and precious advices. A special thank you to Sally Roberts for discussions, experimental advices and valuable comments on my thesis.

A big thank you to present and past members of the Tumour Virology Laboratory:

Paola Massimi, for scientific discussions, technical advice and for having dragged me for lunch every day during the preparation of the thesis.

Vjekoslav Tomaić, for always being a good buddy in and out of the lab, for discussions, dinners, trips, conferences and for the support provided especially during the writing of the thesis.

Siaw Shi Boon, for tolerating me in the bay, always providing good company during the week ends spent in the lab and for the only, but I bet the best, ginseng soup I ever tried.

Martina Bergant, for helping me to see always the positive side of every situation.

David Pim, for being our constant source of inspiration, for his hilarity and great company during conferences.

Justyna (Karolina), for being a constant source of chocolate, to be always smiling and to be always more stressed than anybody else in the lab.

Ketaki Ganti for excellent food provided during Indian festivals, and, after many complaints, to keep her busy phone in the silent mode while being in the office.

My appreciation goes also to Nisha Narayan , Noor Gammoh and Daniela Gardiol for the great time spent together when they were in the lab, and to Kazunori Nagasaka who was always a good friend and a great buddy during conferences.

Thanks also to Joaquin Manzo Merino (Hakim) and Florencia Facciuto for the good fun.

A special thank also to Vanitha Krishna Subbaiah, last but not the least, for constant encouragement, now also from Singapore, for all the great moments spent together, palacinke, cycling, trips, fighting and all the rest!

I would also like to acknowledge ICGEB for having given me the possibility to perform my PhD studies and the whole ICGEB community to create such a great working environment.

To all my friends for having always a source of distraction and amusement; for barbecues, trips, osmizze, "cavrade" and for all the crazy and deleterious nights spent together.

My appreciation also goes to the members of the Aquarium Club and to the Fit Box team.

Contents

List of Illustrations

Abstract

It is clear that the targeting of PDZ-containing substrates by E6 is important for the normal viral life cycle and for the progression to malignancy. However, which of these PDZ domain-containing proteins is relevant for HPV pathology is still elusive. In this study, we provide the evidence that different PDZ domain-containing proteins are differentially targeted by E6. With these experiments, we identified MAGI-1 as a sensitive proteolytic substrate for both the HPV-16 and HPV-18 E6 oncoproteins. We show that E6 promotes the degradation of membrane-bound and nuclear pools of MAGI-1, and the silencing of E6 expression resulted in the MAGI-1-mediated junctional recruitment of ZO-1. Using a mutant MAGI-1, resistant to E6-mediated degradation, we also show that its expression in HeLa cells also promotes membrane recruitment of the tight junction-associated proteins ZO-1 and PAR3, represses cell proliferation and promotes apoptosis. These findings suggest that E6-mediated inhibition of MAGI-1 function perturbs tight junction assembly, with concomitant stimulation of proliferation and inhibition of apoptosis. We also found that the cell polarity regulator hScrib is differentially targeted by HPV-16 and HPV-18 E6-mediated degradation. Surprisingly, we found that residual levels of hScrib expression are required for the maintenance of high levels of HPV-18 E6 expression in HeLa cells. This is not due to an effect on E6 stability or transcription, but rather is due to an effect upon E6 translation. We provide evidence that hScrib and E6 both regulate the PI3K/mTORC1 pathway, and that hScrib might regulate cap-dependent translation through the modulation of the mTORC1 effector S6 kinase. This provides an unexpected role for hScrib in the regulation of tissue homeostasis, and provides further evidence that E6, by fine-tuning the levels of expression of its different cellular substrates, can impact upon a wide range of biological processes implicated in the pathogenesis of cervical cancer.

Introduction

Oncogenic viruses: proto-oncogenes and tumor suppressors

The term oncogenic virus, or oncovirus, refers to a virus with a DNA or RNA genome whose infection is associated with cancer. This terminology originated from studies of acutely transforming retroviruses in the 1950–60s, however the notion that tumor development could have a viral etiologic origin was postulated at the dawn of the 20th century. The first suggestions that human and animal tumors could be transmitted by viruses came from the observations made by Giuseppe Ciuffo in 1907 and Ellermann and Bang in 1908, in which human warts and chicken leukemia could be transmitted to healthy recipients of cell-free filtrates derived from diseased donors (Ciuffo, 1907; Javier and Butel, 2008). Soon after, in 1909, similar experiments led Peyton Rous to discover that avian sarcomas could be transmitted using filtered cell-free tumor extracts, suggesting that a biological agent, the Rous sarcoma virus (RSV), could transmit a genuine cancer, similar to mammalian solid malignant tumors (Rous, 1910, Rous, 1911). Nevertheless, the concept of a infection-associated origin of cancer was dismissed for long time by the scientific community, and the field of tumor virology gained new interest only between 1930s-1960s with the identification of mammalian oncogenic viruses. In 1933, Richard Shope and co-workers reported the isolation of the first mammalian DNA tumor virus. They revealed that a filterable agent, the Shope papillomavirus, could transmit cutaneous warts in cottontail rabbits (Shope and Hurst, 1933), and subsequently the Shope papillomavirus, now known as cottontail rabbit papillomavirus (CRPV), could produce skin carcinomas when inoculated in domestic rabbits (Rous and Beard, 1934; Syverton and Berry, 1935). In addition, cancer-causing CRPV infections in domestic rabbits, were associated with a failure to produce viral progeny. This observation defined a paradigm for small DNA tumor viruses in which deregulation of the viral life cycle is often the causative event associated with the malignant progression of the infection.

After the first evidence suggesting that papillomaviruses might cause malignant tumors in rabbits, an additional 40 years were necessary for the formulation of a hypothesis speculating on a possible role played by human papillomaviruses HPVs in the onset of genital cancers (zur Hausen *et al.*,

1974; zur Hausen, 1975). This stimulated additional interest in papillomaviruses, which ultimately led in the late 1970s to the identification of cytological changes in cervical smears as being papillomavirus-specific (Meisels and Fortin, 1976). Subsequently in the early 1980s the complete nucleotide sequence of two human papillomaviruses, HPV 1a and 6b, (Danos *et al.*, 1982; Schwarz *et al.*, 1983) and of one bovine papillomavirus, BPV 1 (Chen *et al* ., 1982), were published. In the same period the genomes of two "high-risk" HPV isolates, types 16 and 18, were cloned from cervical cancer biopsies (Durst *et al.*, 1983; Boshart, 1984), providing the first evidence for the presence of these virus types in genital cancers, unlike "low-risk" types, such as HPV-6 and 11, which are mainly associated with genital warts that do not progress to malignancy. In the following years, fundamental insights into HPV-linked carcinogenesis were provided by the demonstration that specific viral genes, such as E7 and E6, were expressed in cancer cells (Schwarz *et al.*, 1985). The first molecular evidence for the transforming potential of papillomaviruses came from studies showing that E6 and E7 could induce immortalization of human keratinocytes (Durst *et al.*, 1987). Finally in 1991 at an International Agency for Research on Cancer (IARC) and World Health Organization (WHO) workshop, the causative role of HPV infection in the onset of cervical cancers was officially recognized by the scientific community (Bosch *et al.*, 1992).

The other two families of small DNA tumor viruses are polyomaviruses and adenoviruses. Polyomaviruses were first discovered in mice (Gross, 1953; Stewart *et al.*, 1953), and this was rapidly followed by the discovery of the simian virus 40 (SV40). SV40 is a naturally occurring infectious agent of the Rhesus macaque (Macaca mulatta) and is not associated with disease in Rhesus macaques or other monkeys. The virus was originally isolated as a contaminant of Rhesus monkey kidney cell cultures used to produce the polio vaccine (Sweet and Hilleman, 1960), and was assigned to the family Polyomaviridae, closely related to the human polyomaviruses BK polyomavirus (BKPyV or BKV), JC polyomavirus (JCPyV or JCV), KI (KIPyV), WU (WUPyV) and Merkel cell polyomavirus (MCPyV or MCV), the latter of which has been recently associated with the development of Merkel cell carcinoma (see below) (Feng *et al.*, 2008; Jiang *et al.*, 2009). Research on SV40 gained interest when it was found that Rhesus monkey kidney cell extracts could produce tumors when injected into newborn hamsters (Eddy *et al.*, 1961) and that the agent

responsible for these tumors was indeed SV40 (Girardi *et al.*, 1962; Eddy *et al.*, 1962). Although these tumors in hamsters did not produce progeny virus, the animals were positive for antibodies against the viral protein large tumor antigen (or T-antigen). Furthermore, the small t antigen (or t-antigen) has been subsequently identified as the second SV40 oncoprotein. Small t-antigen possesses a weak transforming activity when expressed alone, but in combination with the T-antigen it cooperates in the induction of transformation (Sleigh *et al.*, 1978; Seif and Martin, 1979). SV40 was also shown to infect human cells and to promote their transformation in tissue culture (Jensen et. al., 1963; Shein, 1967). However, the possible causality between SV40 infection and cancer in humans, particularly in human mesotheliomas and brain tumors, has long been a matter of debate (Fang *et al.*, 2011), and, at present, there is minimal evidence for SV40 implication in the etiology of these tumors. As mentioned before, MCV is the only polyomavirus associated with carcinogenesis in humans, and MCV DNA is found in about 80% of Merkel cell carcinoma (MCC). Although MCV infection of the skin is very common during childhood, MCC occurs primarily in the elderly, and is strongly associated with immunosuppression (Moore and Chang, 2010). Loss of immune control of the infection, promotes a strong reactivation of the replicative life cycle of the virus, and during progression to MCC this event is commonly followed by the viral DNA integration into the host genome (Feng *et al.*, 2008). All MCC cells carry integrated MCV DNA molecules, suggesting that integration is a crucial event for MCC development. Viral DNA integration is believed to result from mutations in the MCV genome arising from the exposure of the skin to mutagenic agents, such as UV radiation. Integration is associated with the loss of viral replicative capacity, however, the expression of large T antigen is maintained and is required for the prolonged survival of MCC cells (Houben *et al.*, 2010). The induction of the fully transformed phenotype is associated with additional mutations in the large T antigen ORF, which results in the loss of its ability to regulate viral DNA replication, but retains the ability to express its differentially spliced forms small and middle T antigens (Shuda *et al.*, 2008; Shuda *et al.*, 2009) and to interact with host cell tumor suppressors, such as pRB (see below).

The third member of small DNA tumor viruses are adenoviruses. These were first isolated in the 1950s from adenoid and tonsil explants, where these viruses were found to be responsible for acute

respiratory diseases (Rowe *et al.*, 1953; Hilleman and Werner, 1954). A few years later, it was shown that infection with some strains of adenovirus could lead to the formation of tumors in hamsters (Trentin *et al.*, 1962). Similar to papillomavirus- and SV40-driven tumorigenesis, tumor cells from infected hamsters did not produce viral progeny and the hamsters developed antibodies against the adenovirus oncoproteins, E1A and E1B.

A number of other viruses are also defined as cancer-causing. These include the herpesviruses Epstein-Barr virus (EBV) and Kaposi's sarcoma herpes virus (KSHV), the hepatitis B (HBV) and C (HCV) viruses and human T-cell leukemia virus-1 (HTLV-1) (Banks *et al.*, 2012). As will be clear from the following discussion, research on the oncoproteins expressed by DNA tumor viruses has led to the discovery of cellular pathways commonly inactivated by viral oncoproteins and deregulated in most human cancers. In addition, they have provided excellent examples of oncogene addiction, since the expression of the oncoproteins in tumor cells is continuously required to maintain the transformed phenotype.

The oncogenes expressed by papillomaviruses, SV40 and adenoviruses act as direct carcinogens. By definition, a genomic sequence coding for a direct viral carcinogen is present in each cancer cell and expresses oncoprotein(s) that directly contribute to cell transformation. Indeed, in studies aimed at defining the viral DNA status in transformed cells, some regions or all of the SV40 DNA was found to be integrated into the host genome (Sambrook *et al.*,1968), whereas only a portion of the Adenovirus DNA was found integrated into host chromosomes (Sambrook *et al.*, 1980). However, in tumors and in transformed cells in culture, the expression of selected genomic regions is maintained. For SV40, this region corresponds to the coding sequence for the large-T and small-t antigens (Huebner *et al.*, 1963), whereas for Adenoviruses this is the early region of the genome expressing the E1A and E1B oncoproteins (Ross *et al.*, 1980a, b). Likewise, in HPV-transformed cells the expression of E6 and E7 is responsible for the establishment and maintenance of the transformed phenotype. The functional relevance for the continued expression of viral oncoproteins became clear when they were found to interact with and inactivate important host cell tumor-suppressors, such as p53 and pRB. The first evidence for this came from the fact that hamsters

carrying SV40-induced tumors possessed high levels of antibodies against a 54 KD protein (Linzer and Levine, 1979), and that the high levels of expression of this protein, p53, in SV40 transformed cells were directly dependent on the expression of the viral T-antigen (Linzer *et al.*, 1979), generating the mistaken concept that p53 might be a T antigen-induced oncoprotein rather than a tumor-suppressor. These observations were soon followed by one of the milestones of tumor virology: the discovery of the interaction between SV40 T-antigen and p53 (Lane and Crawford, 1979). However, the importance of this association only became clear much later, when p53 was finally recognized as a tumor-suppressor. At the time of its discovery, the hypothesis that p53 could be a putative oncoprotein was further reinforced by recent discoveries suggesting that activating mutations in cellular proto-oncogenes could be a common mechanism during cell transformation (Parada *et al.*, 1982; Der *et al.*, 1982; Sukumar *et al.*, 1983). In addition, cDNA clones coding for p53 generated in the early 1980s contained a mutation at codon 135 that conferred transforming capacity on the protein (Levine *et al.*, 2004). It was only several years later that the comparison of the transforming p53 clones with wild type mouse p53 led to the identification of the mutation, and p53 was rapidly recognized as a potent tumor suppressor by its ability to repress transformation in cultured cells (Hinds *et al.*, 1989; Finley *et al.*, 1989). Moreover, in the same period mutations in the p53 genomic locus were found in colon carcinoma cases (Baker *et al.*, 1989).

Now it is clear that mutations of p53 are associated with over half of all human cancers and its tumor-suppressor potential relies on its ability to promote apoptosis, cell cycle arrest and cellular senescence in response to proto-oncogenic cues, such as oncogene-induced hyperproliferation or DNA damage (Kastan and Bartek, 2004; Bieging and Attardi, 2012). The inactivation of p53 by viral oncoproteins was suggested to be a common tumorigenic mechanism used by small DNA tumor viruses, and this was supported by the observation that adenovirus E1B was also able to form a protein complex with p53 in adenovirus-transformed cells (Sarnow *et al.*, 1982). Subsequently, the E6 oncoproteins expressed by HPV-16 and -18 were found to complex with p53 (Werness *et al.*, 1990), but unlike adenovirus and SV40 oncoproteins, E6 recruited a host E3-ubiquitin ligase, E6AP, to promote the proteasome-mediated degradation of p53 (Scheffner *et al.*, 1990). The requirement by DNA tumor viruses to inactivate a potent pro-apoptotic protein such as

p53, is a direct result of their replicative life cycles, where the virus promotes S-phase re-entry and therefore stimulates high levels of DNA replication. This is accomplished through the inactivation of members of the "pocket protein" family, which includes the product of the retinoblastoma tumor susceptibility gene, pRB and its related proteins p107 and p130. The best studied functions of the pocket proteins are their abilities to repress transcription of E2F-responsive genes, through the direct association with members of the E2F family of transcription factors. Phosphorylation of pocket proteins by G1 cyclin-dependent kinases promotes the release of E2Fs, triggering the activation of genes required for cell cycle progression and proliferation (Reviewed in Manning and Dyson, 2011). The tumor suppressor activities of p53 and pRB are integrated by the expression of two other potent tumor-suppressor genes, $p16^{INK4a}$ and $p14^{ARF}$, both expressed by the INKa/ARF locus (Sherr and Weber, 2000). The potential genome instability originating from loss of pRB function is prevented by the activation of p53 which, in turn, leads to cell-cycle arrest through activation of the cyclin-dependent kinase (CDK) inhibitor $p21^{cip1}$ and repression of cyclin B and CDK1 expression (el-Deiry *et al.*, 1993; Smits and Medema, 2001). This is achieved by a feedback loop leading to the expression of $p14^{ARF}$, which induces the activation of p53 by inhibiting MDM2, the ubiquitin ligase responsible for the degradation of p53 (Stott *et al.*, 1998). In addition, $p14^{ARF}$ also promotes the hAda3-mediated acetylation of p53, ultimately leading to cellular senescence (Sekaric *et al.*, 2007). On the other hand, loss of p53 function and induction of oncogenic stress triggers the expression of $p16^{INK4a}$, a potent inhibitor of cyclin-D-dependent kinases that prevents the inhibitory phosphorylation of pRB (Serrano *et al.*, 1993; Suzuki-Takahashi *et al.*, 1995; Sherr and Weber, 2000). In agreement with these activities the INKa/ARF, p53 and pRB genomic loci are among the most commonly mutated genes in human cancers (Sherr and Weber, 2000), and DNA tumor viruses have evolved efficient mechanisms to overcome their tumor-suppressor activity (Shamanin *et al.*, 2008). Figure 1 summarizes how the activities of p53 and pRB are interconnected and their interplay in the prevention of cancer development.

The Rb protein was shown to interact with SV-40 large T-antigen (DeCaprio *et al.*, 1988), adenovirus E1A (Whyte *et al.*, 1988) and HPV E7 (Dyson *et al.*, 1989; Boyer *et al.*, 1996). HPV E7, E1A and SV40 T-antigen share sequence homology in three regions, designated conserved

region (CR) 1, 2 and 3 (Dyson *et al.*, 1992). Strikingly all three viral proteins harbor a short amino acid stretch (LXCXE) lying within the CR-2 region allowing for the interaction with members of the pocket protein family (Dyson *et al.*, 1992; Helt *et al.*, 2002). Interaction of SV40 T-antigen and adenovirus E1A with pocket proteins results in their displacement from E2F transcription factors (Bagchi *et al.*, 1990; DeCaprio *et al.*, 1988; Dyson *et al.*, 1989), and possibly also in the alteration of their phosphorylation status (Wang *et al.*, 1991; Parreno *et al.*, 2001). Conversely, E7 oncoproteins deriving from high-risk HPV types drive the proteasome-mediated degradation of pRB, p107 and p130 (Boyer *et al.*, 1996; Helt *et al.*, 2001; Gonzalez *et al.*, 2001). Whilst the pRB binding is a conserved feature among high- and low-risk E7 proteins, and all are able to interact with the pocket proteins, only those E7s derived from high-risk viruses can direct proteasome-mediated degradation of Rb. In the case of p130, this is bound and targeted for degradation by high- as well as low-risk E7 oncoproteins (Felsani *et al.*, 2006; Klingelhutz *et al.*, 2012), suggesting that the pocket protein-binding motif is necessary but not sufficient for the ability of E7 to drive their degradation.

In the context of HPV- and adenovirus-mediated tumorigenesis, E7 and E1A posses respectively the strongest oncogenic potential. Consistent with this, the expression of E1A alone is able to promote cellular immortalization (Houweling *et al.*, 1980), whereas E1B lacks transformation activity (Van den elsen *et al.*, 1983), but E1A and E1B together cooperate to transform rodent cells (Graham., 1984). Similarly, HPV E7 possesses the stronger transforming activity, but together E6 and E7 cooperate to transform human keratinocytes, the natural host of HPV infection (Barbosa and Schlegel, 1989; Hawley-Nelson *et al.*, 1989; Münger *et al.*, 1989a; Watanabe *et al.*, 1989).

It is clear that inactivation of p53, pRB and pocket proteins is common in the life cycles of human tumor viruses, and that is reflected in the tumors associated with their infection. It is also clear that additional functions of viral oncoproteins, independent of the p53 and pRB targeting, are important for the regulation of viral life cycle and tumorigenesis. For instance, high-risk HPV E6, adenovirus 9 E4ORF1 and HTLV-1 Tax evolved the ability to interact with PDZ domain-containing proteins (see below), and this was shown to play crucial roles in context of the viral life cycles and oncogenic transformation (Banks *et al.*, 2012).

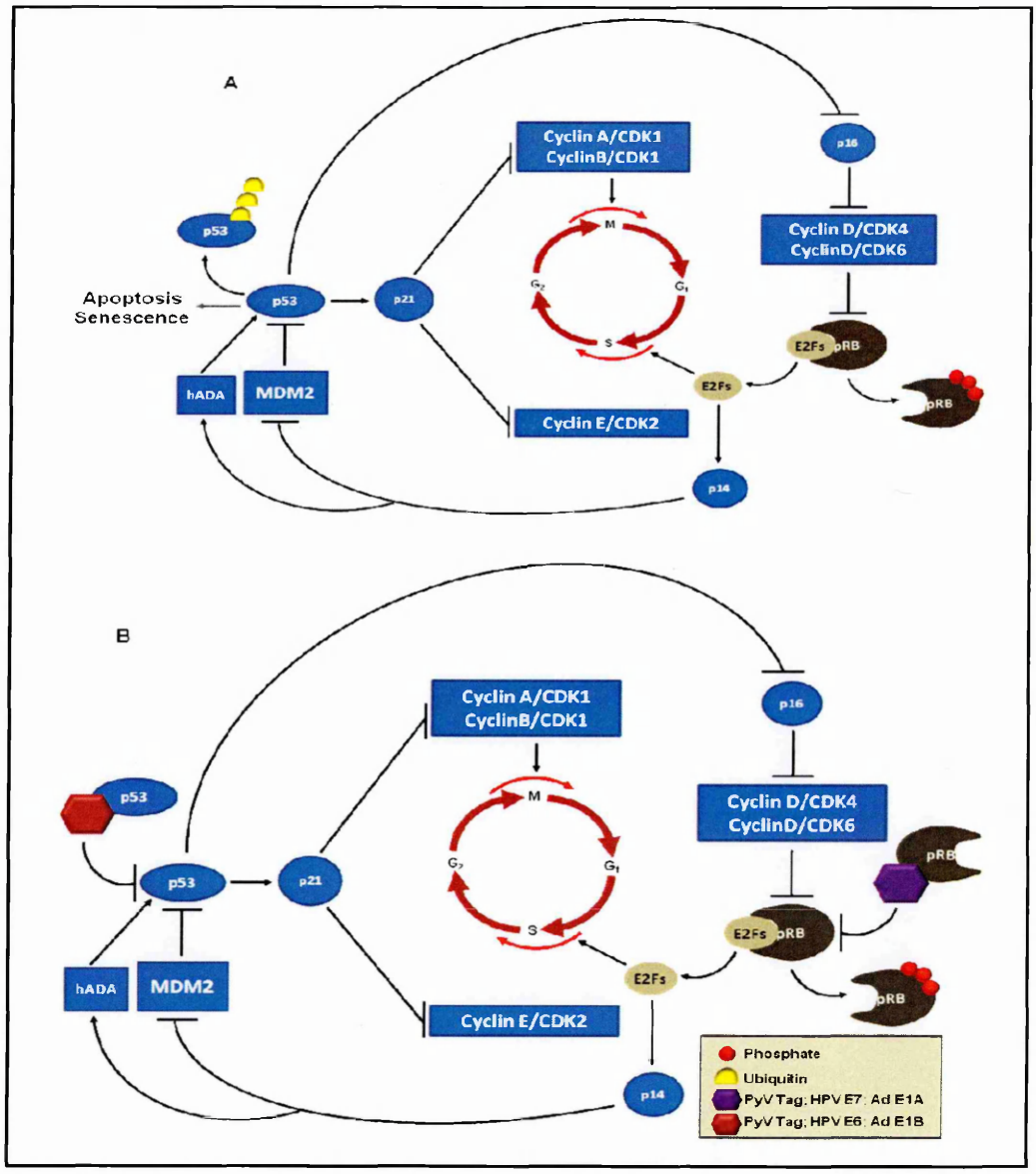

Figure 1. Cartoon showing the interconnections between p53 and pRB pathways and their inactivation by small DNA tumor viruses. A. the expression of $p14^{ARF}$ and $p16^{INKa}$ is part of a feedback loop that integrates p53 and pRB tumor-suppressor pathways. The activation of either $p14^{ARF}$ or $p16^{INKa}$ following loss of pRB or p53 respectively, activates mechanisms leading to cell cycle arrest, senescence and apoptosis. B. The expression of the oncoproteins encoded by small DNA tumor viruses leads to the inactivation of both p53 and pRB, thereby inactivating the p14/p16 feedback loop.

The demonstration that certain human tumor virus oncoproteins can target PDZ domain-containing substrates provided an exciting early indication of their potential relevance in the development of human tumors. One of the first suggestions that PDZ-binding motifs (PBMs) (see below) might confer oncogenic potential and, by implication, that their PDZ domain-containing substrates might have tumor-suppressor potential, came from studies in a mouse model of mammary tumorigenesis. In this system, adenovirus 9 (Ad9) E4ORF1 protein, has potent transforming activity, and this was dependent on an intact PBM (Lee *et al.*, 1997). The E4ORF1 oncoprotein is expressed by the E4 region of the adenovirus genome, which encodes viral proteins important for the regulation of viral DNA replication and late gene expression (Halbert *et al.*, 1985). The Ad9 possesses a unique tumorigenic capacity, and, unlike other adenoviruses, is able to induce estrogen-dependent mammary tumors in mice (Ankerst and Jonsson, 1989; Javier *et al.*, 1991), and this phenotype is conferred by the E4ORF1 PBM (Weiss and Javier, 1997; Thomas *et al.*, 1999; Thomas *et al.*, 2001). The precise contributions of specific PDZ domain-containing substrates to this activity have remained elusive, although hDlg and MAGI-1 appear to be good candidates as tumor suppressors in this system (Javier, 2008). A particularly interesting development was the realization that cancer-causing HPVs also encode a class-1 PDZ binding motif at the extreme C-terminus of the E6 oncoprotein (Kiyono *et al.*, 1997; Lee *et al.*, 1997; Massimi *et al.*, 2004). This is a highly conserved feature among the high-risk mucosal HPVs, such as HPV-16 or HPV-18, and this motif is absent from those HPV E6 proteins that are only associated with benign lesions. In addition, an intact PBM on E6 is essential for many of E6's associated activities, including the regulation of viral life cycle, induction of EMT (epithelial-to-mesenchymal transition) and induction of malignancy in transgenic mouse models (Thomas *et al.*, 2008). Another human tumor virus, HTLV-1, the causative agent of adult T-cell leukaemia, also encodes an oncogene, Tax, which has a PBM, through which it interacts with hDlg (Lee *et al.*, 1997). Unlike E6, Tax does not seem to be involved in the later stages of disease, but an intact PBM does seem essential for the capacity of Tax to transform cells (Tsubata *et al.*, 2005; Higuchi *et al.*, 2007), suggesting that PBM–PDZ interactions might contribute to tumor initiation by HTLV-1.

PDZ proteins in homeostasis and disease of epithelial tissues

PDZ domains and PDZ-binding motifs

PDZ (PSD-95, Disc-Large, Zonula-Occludens-1) domains are named based on the names of the first proteins in which the domain was recognized (Songyang *et al.*, 1997). PDZ domains are motifs of 80-90 amino acids that form a conserved pattern of tertiary structures composed of 6 β-sheets (βA-βF) and two α-helices (αA-αB). These are shown as a cartoon in Figure 2a, aligned with each of the three PDZ domains of human hDlg. Linear amino-acid stretches form joining loops that connect the different structural components of the PDZ domain. The shape of a correctly folded PDZ domain 2 of human hDlg is shown in Figure 2b. PDZ domains interact with PBMs located most commonly at the C-terminus of the target proteins, although internal PBMs also exist, and the specificity is given by the interaction between ligand side chains and the PDZ domain itself (Harris *et al.*, 2001). Early studies identified two classes of PDZ domains based on the ligand specificity displayed: class I PDZ domains target sequence X[T/S]XΦCOOH, and Class II PDZ domains target sequence XΦXΦCOOH, where X is any residue and Φ is an hydrophobic amino acid (Songyang *et al.*, 1997; Nourry *et al.*, 2003). Less common PDZ domain-binding specificities were also identified with the class III PDZ domains (X[ED]XΦCOOH) (Stricker *et al.*, 1997). More recent studies, however, have shown that PDZ domains can interact with up to seven residues in the PBM (Zhang *et al.*, 2007; Thomas *et al.*, 2008), and this elicited the further subdivision of PDZ domains into 16 different subclasses (Tonikian *et al.*, 2008; Subbaiah *et al.*, 2011). Therefore, a single PDZ domain can interact with a multiplicity of different PBMs, making the number of possible PDZ-ligand combinations incredibly high. Target PDZ-binding peptides lodge in a cleft between βB and αB strands, forming a hydrogen bonding network that allows the C-termini of target proteins to interact with the carboxylate binding loop connecting the βA and βB sheets (Jemth and Gianni, 2007). This loop contains the consensus sequence R/K-X-X-X-G-Φ-G-Φ, also known as the GLGF repeat, that facilitates the hydrogen bonding to carboxylate groups and provides the specificity for the PDZ-binding sequence. An example of the mode of interaction of HPV-18 E6 PBM with the PDZ domain 2 of hDlg is shown in Figure 2c. The dissociation constants

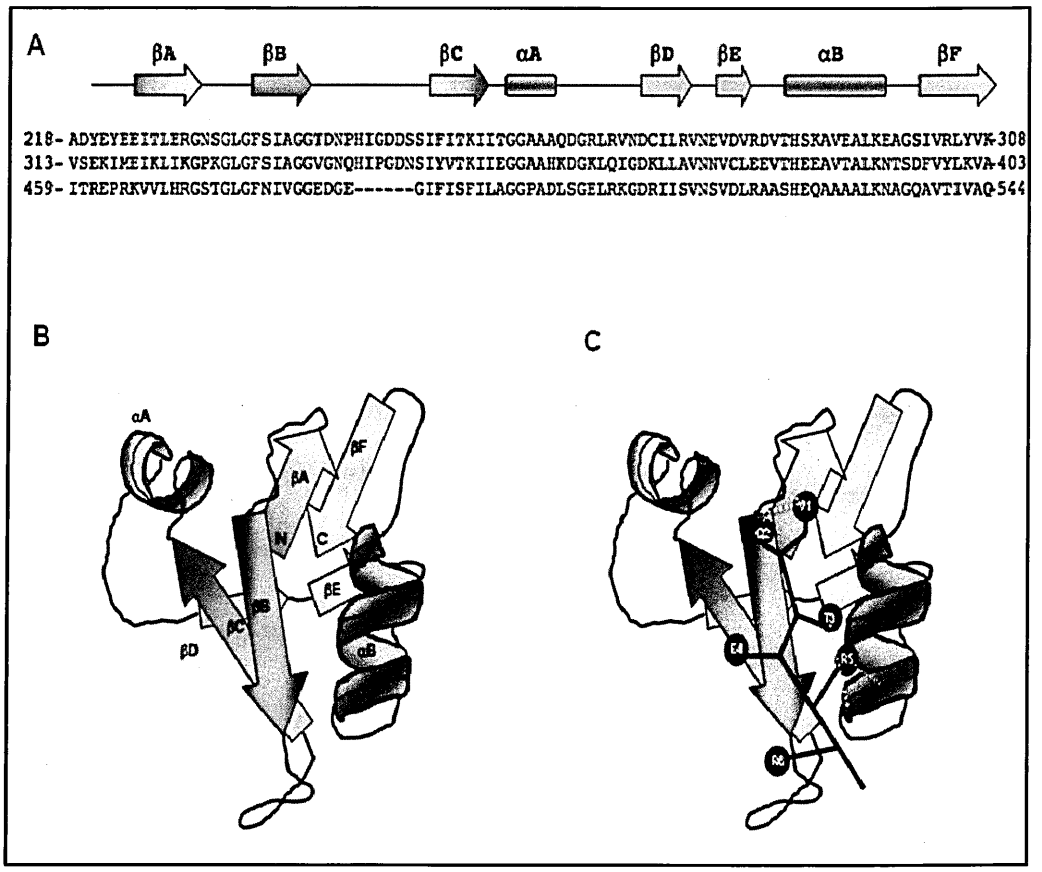

Figure 2. Structural features of PDZ domains. A. A cartoon in linear format of the structural elements that compose a PDZ domain (adapted from Junqueira *et al.*, 2003) aligned with each of the three PDZ domains of human hDlg (hDlg). B. A cartoon showing the folding of the structural elements in Figure 2a, based on the crystal structure of hDlg PDZ2 (Zhang *et al.*, 2007). The proximity of the N and C termini can be seen. (C) A cartoon showing the binding of a PDZ binding motif (PBM), in this case the 6 C-terminal amino acids of HPV-18 E6, to the PDZ domain of hDlg (Zhang *et al.*, 2007). The antiparallel binding of the peptide can be seen.

(K_ds) calculated in solution for the α1-syntrophin PDZ domain and PSD-95 PDZ domains 2 and 3 were in the low micromolar range (1-10 μM) (Niethammer *et al.*, 1998; Harris *et al.*, 2001). This moderate affinity places PDZ-mediated interactions in the same range as SH2 and SH3 domains, and suggests that these interactions are highly reversible in cells and are likely to be subject to regulatory mechanisms (Nguyen *et al.*, 1998). In the case of PDZ-PMB associations, the on/off switch appears to be regulated in some cases through phosphorylation events (reviewed in Jeleń *et al.*, 2003; Kim and Sheng, 2004). Multiple kinase pathways have been shown to regulate PBM-PDZ interactions, indicating that a variety of biological processes are mediated through PDZ domains. In most cases, phosphorylation of phospho-acceptor sites in close proximity to the PBM negatively affects the binding of the PBM to the PDZ target. For instance, phosphorylation of the high-risk E6 PBM by PKA and Akt has been shown to prevent its interaction with PDZ-containing substrates (Kühne *et al.*, 2000; Boon and Banks, 2013), and similarly phosphorylation events within the PBMs of the inward rectifier K^+ channel (Kir2.3), β2-adrenergic receptor and glutamate receptor (GluR2) have been shown to negatively regulate their PDZ-mediated recognition (Cao *et al.*, 1999; Matsuda *et al.*, 2000). However, examples exist in which the regulatory phosphorylation events occur in the PDZ domain, and in this case it promotes the interaction with the PBM (Hegedus *et al.*, 2003). PDZ domain-containing proteins can generally be subdivided into one of three groups according to their domain composition: 1) PDZ-only proteins, containing exclusively one or multiple PDZ domains; 2) membrane associated guanylate kinases (MAGUKs) which contain one or multiple PDZ domains along with a SH3 and guanylate kinase (GUK) domains; and 3) PDZ-proteins containing additional domains (reviewed in Jeleń *et al.*, 2003; van Ham and Hendriks, 2003).

Examples of some of the PDZ domain-containing proteins belonging to the three classes are depicted in Figure 3. Many PDZ domain-containing proteins typically function as scaffolds to recruit soluble proteins and assemble macromolecular signaling complexes at specialized cell-to-cell contact sites termed adherens junctions (AJ) and tight junctions (TJ) (Javier *et al.*, 2008).

Epithelial polarity complexes and establishment of apico-basal cell polarity

Epithelial cell polarity can be referred as the establishment of asymmetries within a cell or a tissue. Cell polarity is required for the regulation of key biological processes and most aspects of development (i.e. organ development and function), and its loss is associated with a large proportion of late-stage cancers (Martin-Belmonte and Perez-Moreno, 2011). In complex eukaryotic tissues, such as stratified epithelia, two types of cell polarity can be distinguished: apico-basal polarity (ABP), in which functional membrane domains are set along the vertical axis of the cell through the polarized distribution of so called polarity complexes; and planar cell polarity (PCP), which refers to the coordinated positioning of cells within the plane of the epithelium. Often components of the ABP mediate pathways involved in PCP and vice versa, therefore the two types of polarity are interdependent and the maintenance of both is a requisite for regulation of cell adhesion and tissue architecture. Increasing evidence suggests that their perturbation is associated with early stages of tumorigenesis and cancer progression (McCaffrey and Macara, 2011; Martin-Belmonte and Perez-Moreno, 2011; Banks *et al.*, 2012). For the purpose of this thesis I will focus on ABP, and in the next section I discuss how polarity proteins set ABP within cells, and provide evidence that link their loss by genetic inactivation or targeting by viral oncoproteins to tumorigenesis.

In vertebrate epithelia, three polarity complexes have been identified: the Crumbs (Crb-PALS1-PATJ) complex, the PAR (Cdc42-PAR6-PAR3-aPKC) complex, and the Scribble (Scrib-Dlg-Lgl) complex. Polarity complexes are formed by polarity determinants that were originally identified in model organisms such as worms, yeast and flies, and their high evolutionary conservation is underlined by the fact that mammalian members of the Scrib complex can complement their counterparts in *Drosophila melanogaster* and yeast mutant cells (Thomas *et al.*, 1997a; Kim *et al.*, 2002; Dow *et al.*, 2003; Grifoni *et al.*, 2004). Cell polarisation is a stepwise process which requires the coordinated interplay of different biological processes, including migration, cadherin-based cell adhesion (see below) and cytoskeleton remodelling.

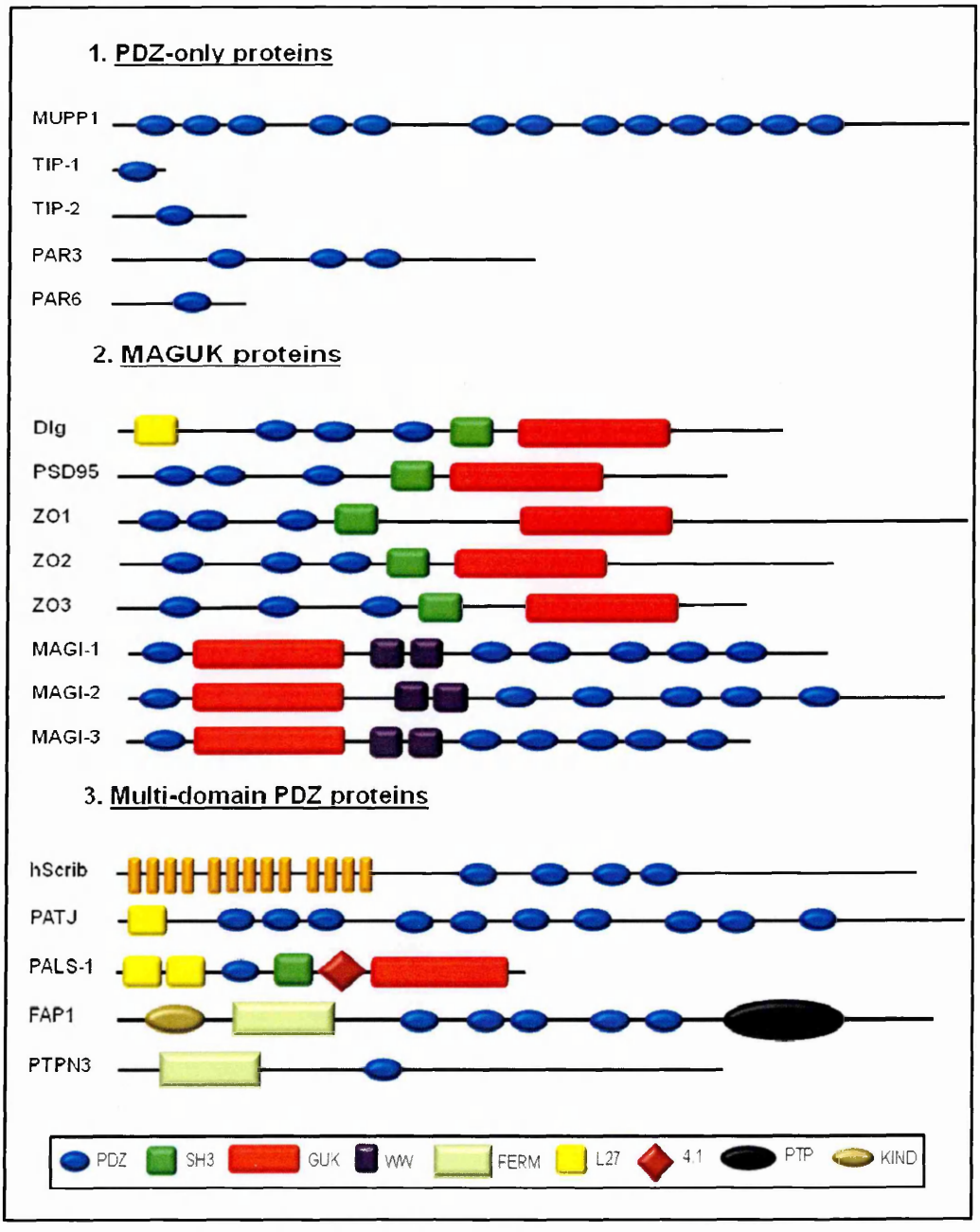

Figure 3. Diagrammatic representation of PDZ domain-containing proteins belonging to three different groups: PDZ-only proteins, MAGUK proteins and multi-domain PDZ proteins.

Figure 4a recapitulates the basic steps leading to cell polarisation. Polarity complexes set cell polarity by distributing asymmetrically in epithelial cells, and by restricting their reciprocal pattern of localisation. In this scenario, the Crumbs complex localizes to, and identifies, the uppermost apical domain which is often associated with the formation of specialized membrane structures, such as microvilli and primary cilia which are connected to actin and spectrin filaments. Conversely, the PAR and the Scrib complexes localize respectively at TJ and AJ (Figure 4b). In vertebrate cells, TJ set the limit between the apical and lateral domains of the cell, where transmembrane proteins, such as junctional adhesion molecules (JAMs), occludin and claudins, form a semi-permeable barrier that limits the paracellular diffusion of solutes. Adherens junctions define baso-lateral membrane identity and assemble beneath the TJs; the respective localizations of these two structures set the limit between the baso-lateral and sub-apical regions of the cell. As their name suggests, AJs represent the main adhesive cellular structures, and are characterized by the presence of cadherins and adaptor proteins such as β- and α-catenin which mediate homophilic interactions between cells (St Johnston and Ahringer, 2010).

PAR complex

The PAR complex includes the PDZ domain-containing proteins partitioning defective (PAR) 3 and 6, the Ca^{+2} and diacylglycerol-independent atypical protein kinase C (aPKC) and the cell division control protein 42 (Cdc42), and it regulates the assembly of TJs. The initial stages of polarization and TJ assembly require the formation of cadherin-based homophilic adhesive structures. These primordial adhesive structures, known as puncta, contain a mixture of AJ and TJ components, including junctional adhesion molecules (JAMs), PAR3 and zonula occludens (ZO)-1 (Suzuki *et al.*, 2002). The subsequent dissociation of AJ and TJ components, and the maturation of separated junctional complexes, requires the activation of the RAC1 GTPase and the kinase activity of aPKC (PKCζ and PKCι in humans). PAR3 directly participates in restricting the activation of RAC1 to primordial adhesion structures through the recruitment of T lymphoma invasion and metastasis-inducing protein 1 (TIAM1), a RAC1 guanine nucleotide exchange factor (GEF), and its exclusion from subapical sites (Chen and Macara, 2005). Subsequently, aPKC is directly involved

in the recruitment of the PAR complex at the TJs through the phosphorylation of PAR3, promoting its PDZ-mediated association with transmembrane TJ protein JAM-1 (Ebnet *et al.*, 2001; Itoh *et al.*, 2001; Hirose *et al.*, 2002), an activity that requires PAR6 to interact with and bring together aPKC and PAR3 (Schneeberger and Lynch, 2004). In addition, PAR6 tethers the PAR complex to the Rho family GTPase, Cdc42, (Joberty *et al.*, 2000; Lin *et al.*, 2000), an interaction that enhances the aPKC kinase activity (Yamanaka *et al.*, 2001), favouring the phosphorylation of additional TJ components, including occludin, claudin-1 and ZO-1, at later stages of TJ assembly (Nunbhakdi-Craig *et al.*, 2002). The activity of aPKC is counteracted by protein phosphatase 2A (PP2A), the first serine/threonine phosphatase found to localize at the TJs, which negatively regulates TJ assembly by dephosphorylating aPKC itself and its TJ substrates (Nunbhakdi-Craig *et al.*, 2002). Therefore, TJ assembly and initial stages of cell polarity are regulated by fine-tuning of the aPKC kinase activity, as well as that of RAC1 and Cdc42 GTPases.

Crb complex

Of the three mammalian homologs of *Drosophila* Crumbs, Crb3 is the one expressed at the apical compartment of epithelial cells (Roh and Margolis, 2003). Crb3 is a transmembrane protein and coordinates the formation of the Crb polarity complex through the cortical recruitment of the MAGUK protein PALS1, mediated by the interaction between the PDZ domain of the latter and the C-terminus of Crb3. In addition to a PDZ domain, PALS1 possesses additional protein-protein interaction modules, including a L27 domain, which promotes the interaction with, and the apical recruitment of, PATJ (Schneeberger and Lynch, 2004), the third component of the Crb polarity complex. The Crb complex is physically linked to TJs through the interaction of PALS1 with PAR6. This association is mediated by the PDZ domain of PAR6 and N-terminus of PALS1 and is enhanced by the GTPase activity of Cdc42 (Hurd *et al.*, 2003). In addition, the C-terminal PBMs of the TJ proteins ZO-3 and claudin-1 bind respectively to the sixth and eighth PDZ domains of PATJ (Roh *et al.*, 2002), strengthening the association of the Crb complex with TJs. The importance of the interaction between the Crb and the PAR complexes for the maintenance of apical polarity identity is underlined by the fact that overexpression of a dominant-negative form of PATJ in

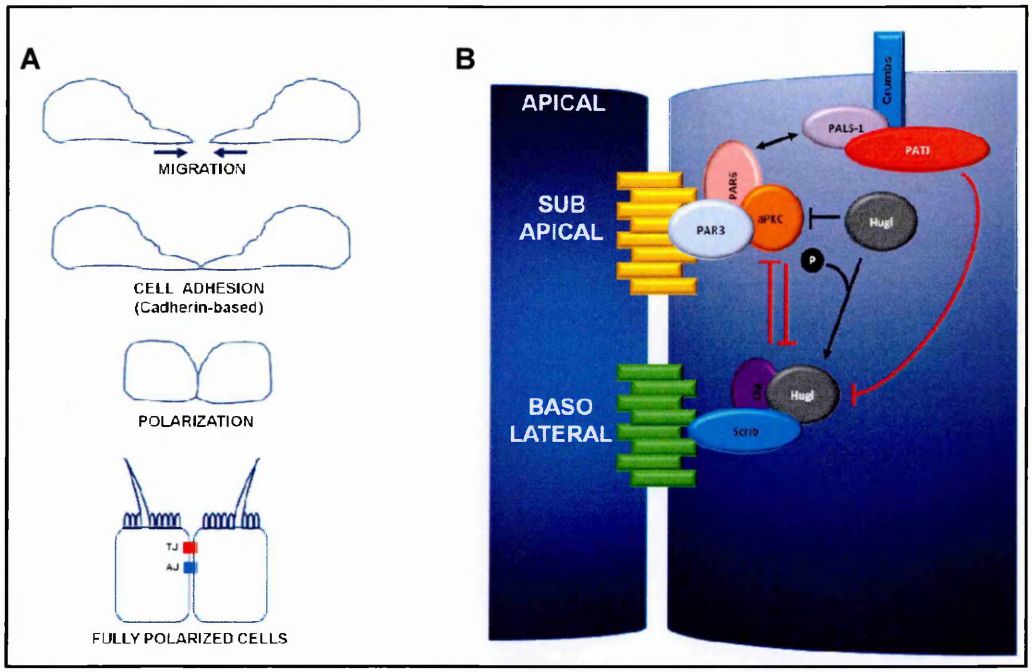

Figure 4. Establishment of cell polarity in epithelial cells. A. Representation of the sequential events occurring during acquisition of cell polarity in epithelial cells (adapted from Ebnet *et al.*, 2004). B. Cartoon showing the polarized distribution of components of the three polarity complexes along the apico-basal axis. The figure also shows the reciprocal inhibition between the three complexes, the key aPKC-mediated phosphorylation of Lgl (Hugl) and the interaction between the Crb and PAR complexes.

epithelial cells not only causes the mislocalization of PALS1, but also disrupts the localisation of the PAR complex and of TJ core components (Hurd *et al.*, 2003).

Scrib complex

The scribble (Scrib) complex is composed of the leucine-rich repeats and PDZ domain (LAP) protein Scrib, the MAGUK protein Dlg and the WD40 domain-containing protein Hugl (the vertebrate homolog of *Drosophila* Lgl). The Scrib complex localizes at the AJs of mammalian cells, whereas in flies it associates with septate junctions, the *Drosophila* homologs of mammalian TJs (Bilder and Perrimon, 2000; Navarro *et al.*, 2005). The Scrib complex co-localises with E-cadherin where it promotes the basolateral identity by preventing the basal expansion of the apical polarity complexes (Yamanaka and Ohno, 2008). This polarity complex was also shown to regulate the expansion of the apical domain at intermediate stages of polarisation. At this stage, Lgl interacts with PAR6 and aPKC, competing for their binding to PAR3 (Yamanaka *et al.*, 2003), thereby inhibiting the PAR complex assembly and TJ maturation. In later stages of polarisation, the extensive aPKC-mediated phosphorylation of Lgl induces its dissociation from the PAR6-aPKC module (Plant *et al.*, 2003). This is a crucial step during the acquisition of ABP, since phosphorylated Lgl is excluded from nascent TJs and is redirected to the AJ-associated hScrib complex, which in turn allows the assembly of the PAR complex and maturation of the sub-apical domain.

Assembly and signalling from junctional polarity complexes

Epithelial sheets are located at the boundaries between anatomical compartments where they strictly regulate the passage of solutes and immune cells without affecting the overall homeostasis of the tissue (Laukoetter and Nava, 2008). In this context, the polarized formation of cell junctions between neighbouring cells, maintains tissue homeostasis by i) forming spatially segregated cell compartments that respond to stimuli coming from different microenvironments, and ii) functioning as a scaffold for the recruitment of regulatory molecules. Consistent with this, loss of

components of AJs as well as of TJs is associated with loss of cell polarity, increased proliferation and acquisition of invasive capacities (reviwed in Martin-Belmonte and Perez-Moreno, 2011). In the next sections I will provide an overview on the control of cell signalling by junction-associated proteins and on how viral oncoproteins can promote tumorigenesis through their perturbation.

Tight junctions

Biochemical data revealed that TJs are supramolecular complexes composed of hundreds of proteins including transmembrane and scaffolding proteins, cytoskeletal components and signalling molecules (Tang, 2006). Within TJs, bundles of transmembrane proteins (such as claudins, occludin and junctional adhesion molecules (JAM1-4) protrude in the intercellular space and contact the TJs of adjacent cells, while cytoplasmic scaffolding proteins interact with the cytoplasmic domain of transmembrane proteins, to stabilize the intercellular connections, assemble signalling complexes and modulate the activity of regulatory proteins. Within the epithelial sheet, intercellular TJ interactions from adjacent cells form a selective barrier for the paracellular diffusion of solutes and macromolecules (e.g. electrolytes and growth factors) controlling normal growth rates and homeostasis of epithelial tissues by generating spatially segregated microenvironments exposed to different proliferative cues (Laukoetter *et al.*, 2007; Tamura *et al.*, 2008; Vetrano *et al.*, 2008). These two functions of TJ transmembrane proteins are not mutually exclusive since controlled paracellular permeability limits cell proliferation, whereas stimulation of proliferation down-regulates adhesive structures (Farkas *et al.*, 2012). A good example of this regulation is the control of proliferation through the segregation of EGF and EGF-like ligands from their receptors (EGFR and ErbB2-4). Epidermal growth factor receptor (EGFR) and ErbB2-4 are members of the type-1 tyrosine kinase receptor family, and are involved in the regulation of physiological processes that require the controlled activation of proliferation and cell migration, such as development, and wound healing. Activation of EGFR and ErbB receptors occurs through their interaction with EGF or EGF-like ligands, which in turn leads to the stimulation of downstream proto-oncogenic pathways (Sweeney *et al.*, 2001). In polarized epithelial tissues, EGFR and ErbB2-4 receptors are spatially segregated from their respective ligands; receptors

localise at the basolateral domain, whereas ligands are present at the apical domain of the cells and in the extracellular fluid covering the surface of certain epithelia (Vermeer *et al.*, 2003; Tsukita *et al.*, 2008). In this context, intact TJs are believed to be the primary barrier to the paracellular diffusion of EGF and EGF-like ligands, and TJ disruption, for instance by mechanical injury of the epithelium, triggers epithelial proliferation and migration downstream of activated EGFR/ErbB receptors, leading to the rapid restoration of the epithelial sealing (Veemer *et al.*, 2003). This barrier function of TJs has been recognized as a protective mechanism, ensuring a rapid restoration of the epithelial barrier function upon tissue damage (Chao *et al.*, 2003). In addition, this also highlights that the maintenance of separated cellular compartments by junctional complexes limits the acquisition of potentially tumorigenic characteristics by epithelial cells. Figure 5 shows the basic structure of stratified epithelia and how TJs might contribute to the maintenance of tissue homeostasis.

The relevance of aberrant activation of ErbB signaling in human cancers has been provided (Hynes and Lane, 2005; Murphy and Morris, 2012), and recently a link between activated ErbB2, disruption of cell polarity and acquisition of carcinogenic properties has been reported (Aranda *et al.*, 2006; Xue *et al.*, 2012). In 3D breast cancer cell models, active ErbB2 mediates loss of cell polarity through the mislocalization of the PAR6-aPKC module, and cooperates with downstream activated Ras/ERK signaling to activate proliferation (Aranda *et al.*, 2006). This is also consistent with other studies which show that mislocalization or overexpression of PAR6 and aPKC are common in many cancers (Aranda *et al.*, 2008). Expression of a PAR6 mutant unable to bind ErbB2 prevented cell polarity alterations in mammary cells, underlining the critical role played by the PAR complex in epithelial homeostasis. Intriguingly, ablation of hScrib in mutant PAR6-expressing cells, restored the polarity defects produced by the ErbB2/PAR6-aPKC interaction (Aranda *et al.*, 2006), suggesting that the PAR and hScrib polarity complexes can modulate cell polarity and prevent tumorigenesis through the regulation overlapping pathways. This is also supported by the observation that both PAR3 and hScrib can regulate Rac, contributing to the control of polarized cell migration by restricting Rac activity (Qin *et al.*, 2005; Xue *et al.*, 2012). In contrast, a pro-oncogenic activity has been indicated for PAR6, with its overexpression being

linked to increased proliferation and also to the induction of TJ breakdown. Furthermore, TGFβ (transforming growth factor β)-induced phosphorylation of PAR6 increases its interaction with the ubiquitin ligase Smurf1 (Smad ubiquitylation-regulatory factor 1), thereby resulting in RhoA degradation and the induction of a more mesenchymal phenotype (Ozdamar *et al.*, 2005). Thus the PDZ domain-containing components of the Par complex can function as promoters and inhibitors of EMT, depending on the balance of PAR3/PAR6 activities (Aranda *et al.*, 2008).

Components of the PAR complex belong to a large group of proteins that contributes to the assembly of a macromolecular complex on the cytosolic side of TJs, referred to as the TJ plaque. The first TJ plaque protein identified was ZO-1 (zonula occludens-1) (Stevenson *et al.*, 1986). ZO-1, with ZO-2 and ZO-3, constitute the ZO protein family that localises at TJs of epithelial cells in a cell density-dependent manner (Gottardi *et al.*, 1996). ZOs share common structural features that place them in the MAGUK superfamily, including multiple PDZ domains, SH3 and GUK domains and proline-rich regions variable in length, and several ZO-interacting partners have been identified (reviewed in Bauer *et al.*, 2010). A crucial function of junctional-localised ZO-1 is the regulation of RhoA-induced proliferation. This is achieved by ZO-1 directly through the SH3-mediated membrane sequestration of the RhoA effector ZONAB/DbpA (Balda and Matter, 2000) and its associated protein CDK4 (Balda *et al.*, 2003), or through the recruitment of cingulin, another TJ plaque protein, that in turn binds and sequesters the RhoA-specific guanine nucleotide exchange factor (GEF) GEF-H1/Lcf (Citi *et al.*, 2009). ZONAB/DbpA is a Y-box transcription factor, whose nuclear translocation stimulates proliferation by activating the gene expression of the G1-associated cyclin D1, PCNA and ErbB2 (Balda and Matter, 2000; Sourisseau *et al.*, 2006), in an active RhoA-dependent manner (Nie *et al.*, 2009). In addition, ZONAB/DpbA recruits the cyclin D1-associated kinase CDK4 to the nucleus (Balda *et al.*, 2003), thus efficiently promoting the G1-S progression through the cell cycle. In agreement with these data, ZO-1 down-regulation is observed in a large proportion of breast cancer cases (Hoover *et al.*, 1998). The control of cell proliferation mediated by the TJ protein ZO-1 is completed by the second member of the ZO family, ZO-2, whose translocation to the nucleus of proliferating cells has been shown to inhibit the transcription of cyclin D1 (Gonzalez-Mariscal *et al.*, 2009), whereas a role in the modulation of cell-cycle-

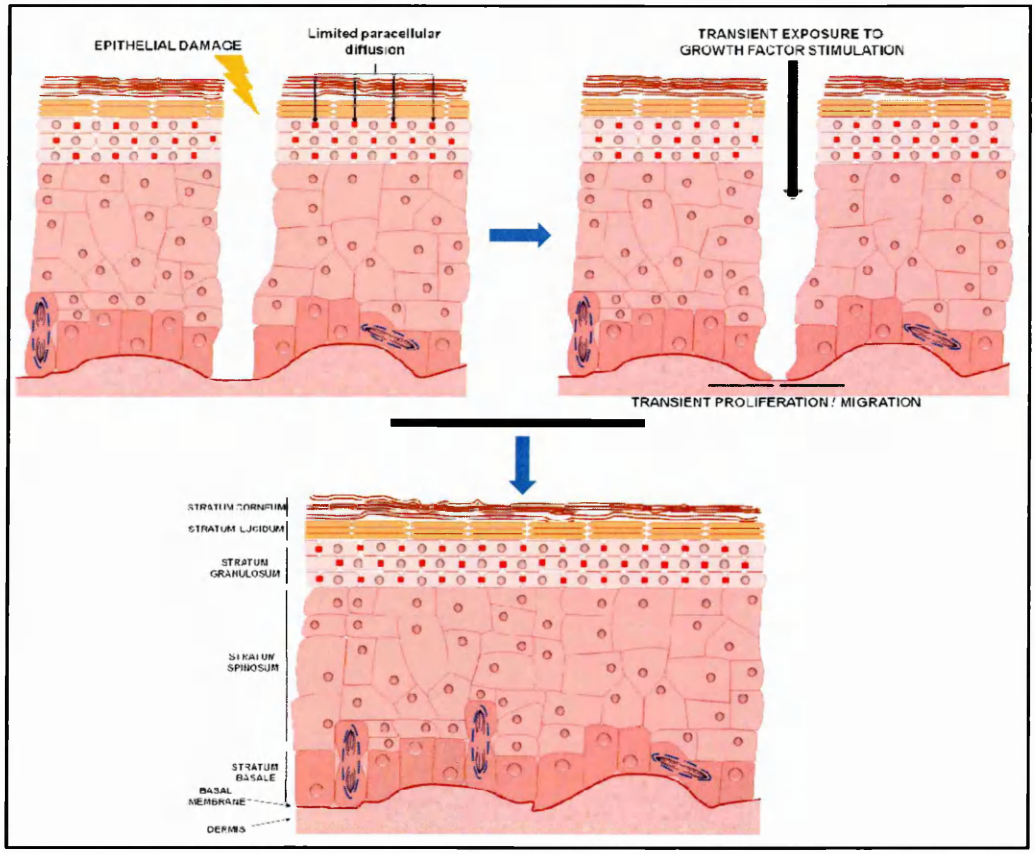

Figure 5. Contribution of TJs to the maintenance of epithelial tissue homeostasis. In stratified epithelia the asymmetric division of basal cells (perpendicular to the basal membrane) generate daughter cells that are pushed along the differentiating epithelium and are committed for terminal differentiation. Cells of the basal layer also maintain the ability to undergo symmetrically cell divisions (parallel to the basal membrane) in order to generate two daughter cells that maintain the stem cell phenotype. In the differentiating epithelium TJs (red squares between cells) are enriched in the granular layer (stratum granulosum) and form a barrier that blocks the paracellular diffusion of solutes and growth factors, thereby preventing the exposure of basal cells to proliferative cues. Upon mechanical injury of the epithelium and TJ disassembly, the lower epithelial layers become exposed to growth factor stimulation which ensures the rapid restoration of the epithelial sealing by promoting transient cell migration and proliferation.

dependent stability of cyclin D1 has been proposed for ZO-3 (Capaldo *et al.*, 2011). The assembly of TJ complexes and the correct localisation of ZO proteins are mutually dependent on each other (Umeda *et al.*, 2006). Thus, in epithelial cells MAGI (MAGUK with inverted domain structure)-1 is recruited to junctional sites by JAM-4 in a PDZ-dependent manner, and this promotes TJ stabilisation and recruitment of ZO-1 (Hirabayashi *et al.*, 2003). MAGI-1 is a member of the MAGI protein subfamily that also comprises MAGI-2 and -3. These are defined as MAGUKs although they differ from the canonical domain composition, having a unique arrangement of protein-protein interaction domains (Dobrosotskaya *et al.*, 1997). MAGI-1 is able to interact with PTEN and β-catenin through its PDZ domains 2 and 5 respectively (Dobrosotskaya and James, 2000; Kotelevets *et al.*, 2005). This set of interactions stabilises β-catenin at the membrane and re-localises PTEN to membrane bound sites, protecting it from proteasome-mediated degradation, a function also found with MAGI-2 (Valiente *et al.*, 2005; Hu *et al.*, 2007). The formation of a trimeric β-catenin–MAGI–PTEN complex has been shown to mediate PTEN tumor-suppressor function in the context of oncogene activation (Kotelevets *et al.*, 2005). This is in agreement with the fact that membrane-bound PTEN down-regulates the PI3K signalling pathway (Leslie and Downes, 2002), thus inhibiting several processes related to tumor formation and cancer progression, including cell growth, survival and migration (Leslie and Downes, 2002; Salmena *et al.*, 2008).

Adherens junctions

Adherens junctions mediate cell-cell adhesion through the formation of homophilic interactions between the extracellular domains of cadherins, which in epithelial cells are best represented by E-cadherin (Nagafuchi, 2001). The adhesive properties of E-cadherin are enhanced by formation of macromolecular complexes, mediated by the direct recruitment of cytosolic proteins by the intracellular domain of E-cadherin. These include β-catenin (the vertebrate homolog of *Drosophila* armadillo), γ-catenin and p120-catenin, which bind directly to E-cadherin and promote its localisation and stability (Reynolds *et al.*, 1994; Yap *et al.*, 1998; Huber and Weis, 2001; Davis *et al.*, 2003). Engagement of cadherin-based junctions represents the basis for the induction of the

polarised epithelial phenotype (Figure 4a). Consequently E-cadherin is, perhaps, the main barrier to the epithelial-to-mesenchymal transition in which epithelial cells lose cell polarity and acquire migratory and invasive capacities. During development, EMT is followed by its reverse process, MET (mesenchymal-to-epithelial transition) to regulate organ morphogenesis. Thus, during malignant transformation in epithelial tissues, the induction of EMT correlates with increased aggressiveness of the tumor (Thiery *et al.*, 2009). So far, several inducers of EMT have been identified; these include the transcription factors TWIST, SNAIL and ZEB-1/2, which directly down-regulate E-cadherin expression through the repression of its promoter activity (Nieto, 2002; Peinado *et al.*, 2007). One of the best known functions of E-cadherin-based AJs in the regulation of tumorigenesis is the control of the Wnt/β-catenin pathway through the modulation of APC (adenomatous polyposis coli) function. APC is a tumor-suppressor and plays a fundamental role in the regulation of the Wnt pathway, orchestrating the formation of the so-called "axin destruction complex", formed by axin, the kinases CK2 and GSK3β, APC and β-catenin, which leads to the recruitment of the E3 ubiquitin-ligase β-Trcp and drives the proteasome-mediated degradation of β-catenin (de Law *et al.*, 2007). Activation of the Wnt pathway and stabilisation of β-catenin, leads to the activation of a number of proto-oncogenic genes (Wnt target genes), whose expression is deregulated in many human cancers (de Law *et al.*, 2007). Consistent with this important tumor-suppressor function, germline mutations in the APC gene are associated with familial adenomatous polyposis (Kinzler and Vogelstein, 1996), a risk factor for development of colon carcinoma. APC has multiple protein interaction sites, one of which includes a C-terminal class 1 PBM (Morais Cabral *et al.*, 1996; Giles *et al.*, 2003). The PBM-dependent interactions have been shown to be crucial for the modulation of its function, and mutations affecting the ability of APC to bind PDZ domains have been found in human cancers (Miyoshi *et al.*, 1992; Pedemonte *et al.*, 1998). The PBM-mediated interaction of APC with hDlg, appears to regulate its correct localization with important consequences for the regulation of cell-cycle progression and cell adhesion (Matsumine *et al.*, 1996; Etienne-Manneville *et al.*, 2005). The APC PBM also binds to the PDZ domain of the FAP-1 (Fas-associated phosphatase 1) tyrosine phosphatase (Erdmann *et al.*, 2000). FAP-1 displays a pleiotropic behaviour in the context of the regulation of tumorigenesis. The phosphatase activity

of FAP-1 is required to inhibit the proliferation of Wnt-stimulated cells through the modulation of the APC/β-catenin complex (Erdmann *et al.*, 2000; Welters *et al.*, 2008); consistent with this, FAP-1 function is lost in colorectal cancers (Wang *et al.*, 2004). On the other hand, FAP-1 had been originally identified for its antiapoptotic function through its ability to de-phosphorylate the death receptor Fas, thereby preventing Fas-ligand mediated apoptosis (Saras *et al.*, 1997; Ungefroren *et al.*, 2001). In addition, FAP-1 expression is progressively increased in cancer progression of the Ewing's sarcoma family of tumors, in which FAP-1 is a direct transcriptional target for EWS-FLI1 (Ewing sarcoma breakpoint region 1/Friend leukaemia virus integration 1) fusion protein (Abaan *et al.*, 2005). Therefore, the function of FAP-1 with respect to tumorigenesis is likely to be highly context-dependent.

As discussed above, AJs are the resident site of the hScrib polarity complex. This complex is essential for regulating cell polarity and proliferation in *Drosophila*, with loss of any component resulting in broadly similar and complementary phenotypes. In human tumors, loss of hScrib and hDlg is a common event in later stages of cancer progression, although at earlier stages of disease progression the two proteins are expressed at extremely high levels and often mislocalised (Watson *et al.*, 2002; Cavatorta *et al.*, 2004; Nakagawa *et al.*, 2004; Gardiol *et al.*, 2006). However, their relative contribution to tumorigenesis in higher eukaryotes has begun to be clarified only recently. Depletion of either hDlg or hScrib in human keratinocytes has opposing effects on cell adhesion, invasion and apoptosis (Massimi *et al.*, 2012). Considering that, in human cells, the function of hDlg still remains to be determined, this study suggested that its function is highly context-dependent. Human hDlg acted as tumor-suppressor during induction of anoikis, an apoptotic pathway induced by the growth of cells in the absence of cell-substratum attachment (Massimi *et al.*, 2012). Conversely, loss of hDlg reduced the invasive potential of human keratinocytes (Massimi *et al.*, 2012), and in a similar study, the depletion of hDlg impaired the invasive capacity of cervical cancer-derived HPV-positive cells (Krishna Subbaiah *et al.*, 2012), suggesting a pro-oncogenic role for hDlg in certain circumstances. This activity of hDlg, however, has been linked to its ability to activate proto-oncogenic pathways upon its mislocalization by viral oncoproteins

(see below) or activated cellular oncogenes (Frese *et al.*, 2006; García-Mata *et al.*, 2007; Krishna Subbaiah *et al.*, 2012).

hScrib is recruited to AJs and cell-cell contacts through its interaction with E-cadherin (Navarro *et al.*, 2005) and the correct localisation of the two AJ components is mutually interdependent (Navarro *et al.*, 2005; Qin *et al.*, 2005). Recent studies have suggested that a central tumor-suppressor function of hScrib is its modulation of the Ras/Raf/MAPK pathway. Loss of hScrib cooperated with oncogenic Ras to promote anchorage-independent growth of breast cancer cells and invasion in organotypic 3D cultures (Dow *et al.*, 2008), although the depletion of hScrib alone was recently shown to be sufficient to promote an invasive phenotype in human keratinocytes (Massimi *et al.*, 2012). In addition, hScrib has been shown to reduce the levels of phosphorylated (active) ERK kinase (Dow *et al.*, 2008; Nagasaka *et al.*, 2010), at least in part, through the recruitment of the cellular phosphatase PP1γ (Nagasaka *et al.*, 2013), and this activity of hScrib is required to suppress oncogenic Ras co-transforming activity (Nagasaka *et al.*, 2010). The tumor-suppressor activity of hScrib also relies on its ability to induce pro-apoptotic pathways (Zhan *et al.*, 2008; Liu *et al.*, 2010). Once again, however, this activity is context-dependent, since anti-apoptotic functions for hScrib have also been reported (Massimi *et al.*, 2012). This is also highlighted by the observation that mislocalization of hScrib is sufficient to promote carcinogenesis in breast cancer cell models through the augmentation of the activity of the Hippo transducer Taz (Cordenonsi *et al.*, 2011).

The transcription factors Taz and Yap are the final targets of the Hippo tumor-suppressor pathway, which is highly conserved from flies to humans (Pan, 2010). The activation of this pathway leads to a signalling cascade that culminates in the sequential activation of the kinases MST1 and LATS1, which in turn leads to the inhibitory phosphorylation of Yap and Taz. The phosphorylation of Taz leads to its proteasome-mediated degradation (Pan, 2010), and in this context hScrib controls the localisation and activity of MST1, which is required for the activation of LATS1. Consistent with this, loss of hScrib expression or its membrane displacement have been shown to induce the

aberrant activation of Taz and acquisition of EMT and cancer stem cell-related traits (Cordenonsi *et al.*, 2011). Similar effects were also mediated by Crb in mouse mammary cells. Assembly of the Crumbs complex resulted in increased Yap/Taz phosphorylation and in the inhibition of TGFβ-SMAD-mediated EMT (Varelas *et al.*, 2010). This suggests that hScrib and Crb could have complementary tumor-suppressor effects through the regulation of the Hippo pathway. hScrib has been implicated in the induction of the c-Myc-mediated apoptotic pathway, through the formation of a ternary complex which includes hScrib, the Rac and Cdc42 GEF βPIX, and the G protein-coupled receptor kinase interactor 1 (GIT1) (Zhan *et al.*, 2008). In polarised breast cancer cells, the c-Myc-mediated hScrib/βPIX/GIT1 complex drives apoptosis through the downstream activation of the Rac-JNK-Jun-Bim apoptotic pathway, whereas loss of hScrib function, caused by RNAi-mediated ablation or by membrane displacement of the protein, redirected c-Myc signalling from a pro-apoptotic to a pro-oncogenic pathway (Zhan *et al.*, 2008), possibly involving an aberrant activation of the MAP kinase JNK (Wu *et al.*, 2010). The interaction of β-PIX with hScrib has also been shown to modulate the correct localisation of the GEF at the leading edge of migrating cells, to promote directional migration by spatially restricting the activities of Rac and Cdc42 (Qin *et al.*, 2005), and to guide epithelial morphogenesis in 3D culture systems through the control of the Cdc42 and Rac effector p21-activated kinase1 (PAK1) (Eastburn *et al.*, 2012). Interestingly, in the latter study hScrib has been identified as a client protein for the HSP90/sgt1 chaperone complex, and the integrity of the HSP90/sgt1/hScrib complex appears to be required to regulate hScrib stability (Eastburn *et al.*, 2012). These data provide molecular evidence for the importance of the correct expression of hScrib for regulation of apoptosis, migration and epithelial morphogenesis.

For the other member of the hScrib polarity complex, the tumor-suppressor properties of Hugl are the least understood. Vertebrates express two homologs of the *Drosophila* Lgl, Hugl-1 and Hugl-2 whose expression have been shown to be deregulated in a variety of human cancers (Schimanski *et al.*, 2005; Kuphal *et al.*, 2006; Tsuruga *et al.*, 2007; Lisovsky *et al.*, 2009). Moreover, recent evidence suggests that Hugl1 and 2 are potent inhibitors of the EMT program. Consistent with this, expression of Hugl-1 and Hugl-2 has been shown to promote junctional E-cadherin localization and re-induction of the epithelial phenotype, respectively, in melanoma and breast cancer cell lines

(Kuphal *et al.*, 2006; Kashyap *et al.*, 2012). Therefore, Hugl proteins are strong inducers of the epithelial program and this is achieved, at least in part, through the modulation of the EMT-associated transcription factor Snail (Kashyap *et al.*, 2012). However, recent data from a zebrafish model for epidermal carcinogenesis, suggest that Lgl2 can maintain the epithelial phenotype by regulating ErbB2-associated signalling (Kashyap *et al.*, 2012). To date there is no evidence for direct targeting of Lgl by oncogenic viruses, however the inactivation of the PDZ-containing members of the hScrib polarity complex are likely to affect also the activity of Lgl.

Targeting of junctional complexes by viral oncoproteins

As noted above, although human oncogenic viruses constitute a highly heterogeneous group of viruses, their life cycles necessitate the modulation of common host cellular pathways (Moore and Chang, 2010) and these effects are reflected in the tumors they produce. One such pathway is cell polarity, and different human tumor viruses adopt overlapping strategies to perturb the expression patterns of polarity proteins, including cadherins and PDZ domain-containing proteins (Figure 6). A list of PDZ domain containing proteins known to be targets for viral oncoproteins is provided in Table 1. Human tumor viruses, including HPV, EBV, HBV, HCV, and KSHV, have all been shown to perturb the pattern of E-cadherin expression. Oncoproteins expressed by EBV, HBV and HCV have been shown to down-regulate E-cadherin expression through the methylation of its promoter or by up-regulating the expression of EMT-associated transcription factors TWIST and SNAIL (Banks *et al.*, 2012). In contrast, KHSV promotes both the degradation and the PAK-1-mediated mis-localisation of VE-cadherin in endothelial cells (Banks *et al.*, 2012). In the case of HPV, both E6 and E7 oncoproteins are able to repress the transcription of E-cadherin by increasing the activity of DNA methyltransferases and E-cadherin promoter methylation (Laurson *et al.*, 2010; D'Costa *et al.*, 2012). In addition, the perturbation of members of the hScrib complex is also likely to affect E-cadherin stability (Qin *et al.*, 2005; Lohia *et al.*, 2012). Thus, HPV and other human tumor viruses perform a coordinated attack aimed at perturbing the pattern of expression E-cadherin, suggesting the importance of inhibiting E-cadherin function during viral life cycles.

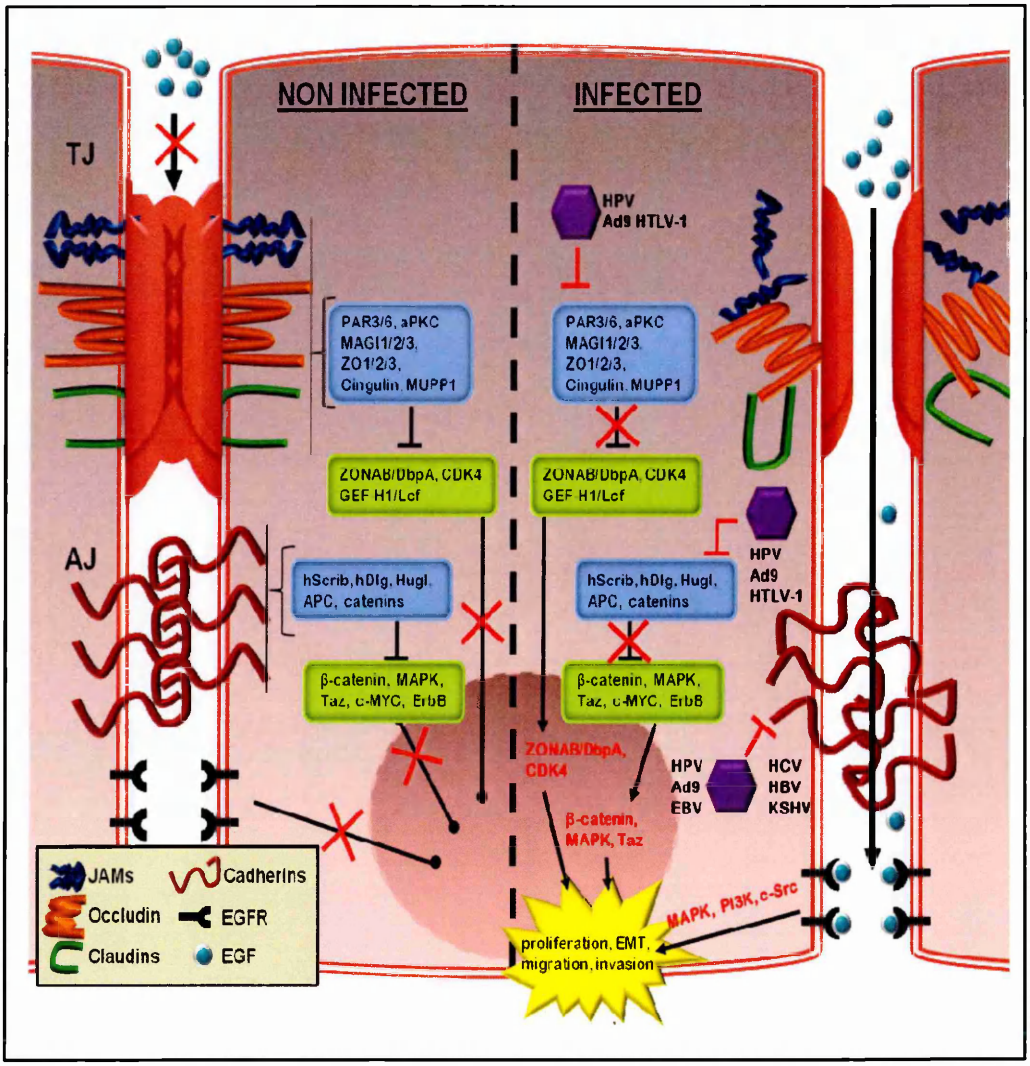

Figure 6. Distribution of polarity and signalling components at the AJs and TJs and their targeting by tumor-associated viruses. See the next page for the full legend.

Figure 6 (Cont.). Depicted are the transmembrane components of AJs and TJs as well as the cytosolic proteins associating with their plaques (blue boxes) and the downstream pathways that are regulated upon their assembly (green boxes) in non-infected and infected cells. The modulation of AJ- and TJ-associated components by oncogenic viruses leads to the deregulation of proto-oncogenic signalling pathways (see text) promoting cell proliferation, migration and invasion. TJ integrity creates separate microenvironments in the basal and apical compartments of polarized cells. Disruption of TJ assembly by oncogenic viruses through the inactivation of TJ-associated cytosolic proteins increases the accessibility of growth factor receptors (e.g. EGFR/ErbB) to their ligands (e.g. EGF), further promoting the activation of oncogenic pathways. Targeting of cadherin-based AJs or its associated proteins contributes to the loss of cell polarity and acquisition of a mesenchymal phenotype.

Besides induction of cell proliferation, another possible role for the perturbation of E-cadherin expression might be linked to its ability to modulate the cell-mediated immune response to viral infection. The recruitment of epidermal antigen-presenting cells to the sites of infection in the epidermis requires the expression of E-cadherin by both antigen- presenting cells and keratinocytes. Loss of E-cadherin expression leads to reduced retention of antigen-presenting cells in the infected tissue (Tang *et al.*, 1993; Jakob and Udey, 1998), and low abundance of antigen-presenting cells at sites of viral infection has been shown to correlate with higher disease severity (Sprecher and Becker, 1983; Sprecher and Becker, 1989). Thus, the targeting of E-cadherin could contribute to the immune evasion by human tumor viruses; however, loss of E-cadherin is also strongly implicated in the process of virus-induced carcinogenesis. As mentioned above, the perturbation of E-cadherin expression is a hallmark of EMT and is invariably associated with loss of APC function and acquisition of invasive properties by tumor cells. In addition, loss of E-cadherin also results in the perturbation of hScrib complex-mediated regulation of cell polarity.

The perturbation in the pattern of hDlg expression has been shown to be associated in some cases with acquisition of tumorigenic capacity, and this, in part, is achieved through the interaction of hDlg with PBM-containing GEFs, including Net1 and SGEF (García-Mata *et al.*, 2007; Krishna Subbaiah *et al.*, 2012). hDlg has been reported to regulate Net1 localization and activity, conversely, oncogenic mutants of Net1 are believed to promote the hDlg sequestration and inhibition of hDlg function (García-Mata *et al.*, 2007). Likewise, high-risk HPV E6 and E4-ORF1 tumorigenic activities have been shown to rely in part on the mislocalization of hDlg. In HPV-18 positive cervical cancer cells, E6 oncoprotein has been shown to recruit hDlg to detergent-insoluble sites where, in cooperation with SGEF, a RhoG-specific GEF, hDlg promotes the invasiveness of HeLa cells via the activation of RhoG (Krishna Subbaiah *et al.*, 2012). The interaction of Ad9 E4-ORF1 with PDZ proteins has been shown to recruit the oncoprotein to cell-cell contact sites, where it drives the activation of the PI3-kinase, an activity that correlates with Ad9 mammary tumorigenesis in mice (Frese *et al.*, 2003). In this context, the E4-ORF1-mediated PI3-kinase stimulation was shown to rely on the formation of a complex with hDlg, resulting in its membrane sequestration where it cooperates with the adenoviral oncoprotein to stimulate the PI3 kinase

pathway (Frese *et al.*, 2006). Another homolog of hDlg, PSD95, has also been identified as a binding partner for high-risk E6 oncoproteins (Gewin *et al.*, 2004). PSD95 is a preferential proteolytic substrate for HPV-18 E6 oncoprotein, and the forced expression of this PDZ protein in HPV-positive cervical cancer cells has been shown to reduce their tumorigenic potential (Handa *et al.*, 2007).

Many PDZ domain-containing proteins of the TJ plaque are inactivated by viral oncoproteins, resulting in the loss of TJ integrity. Consistent with the importance of ZO proteins for the modulation of cell proliferation, ZO-2 was found to interact with adenoviral E4-ORF1 in a PDZ-dependent manner, leading to aberrant cytoplasmic sequestration of the PDZ protein (Glaunsinger *et al.*, 2001). Cells that lack ZO-1 and ZO-2 fail to form TJs (Umeda *et al.*, 2006), and although the targeting of ZO-2 by E4-ORF1 proved the importance of ZO proteins for adenovirus 9-mediated transformation, other members of the ZO family do not appear to be directly targeted by virus-encoded oncoproteins. However, indirect effects are likely, through the inactivation of factors involved in the regulation of ZO localisation/activity. The PDZ proteins MAGI-1 and PATJ, have been shown to be substrates for high-risk HPV E6 and E4-ORF1 oncoproteins (Glaunsinger *et al.*, 2000; Latorre *et al.*, 2005; Storrs and Silverstein 2007), and their inactivation is associated with mislocalisation of ZO-1 and other TJ components (Latorre *et al.*, 2005; Kranjec and Banks, 2011). In the context of HTLV-1-mediated transformation, populations with reduced expression of MAGI-1 are selected during neoplastic transformation (Makokha *et al.*, 2012). In addition, the loss of MAGI-1 observed in acute lymphoblastic leukaemia (Kuang *et al.*, 2008) suggests that its tumor-suppressive potential goes beyond the regulation of junctional stability. In addition, MAGI-2 and MAGI-3 have also been shown to be the target for viral oncoproteins (Thomas *et al.*, 2002; Ohashi *et al.*, 2004) suggesting a potential tumor-suppressor role also for these proteins.

It is interesting to note that the crystal structures of HPV-18 E6 bound to the PDZ domains of MAGI-1, MAGI-3 and hDlg have recently been solved, and this allowed us to define the relative contributions given by single amino acids within the PBM and PDZ domains to the interaction (Zhang *et al.*, 2007; Thomas *et al.*, 2008a). Importantly, these studies revealed that not only

residues within the HPV-18 E6 PBM (ETQV), but also those immediately upstream, contribute to the binding to PDZ proteins. The residues of the PBM specifically contributing to the binding to hDlg, MAGI-1 and -3 varied according to the PDZ substrate considered, suggesting that subtle differences within the PDZ domains of these proteins modulate the interaction with E6 PBM (Thomas *et al.*, 2008a). Moreover, subsequent studies identified the specific residues within the PDZ domain-1 of MAGI-1 that mediate its association with the E6 PBM (Fournane *et al.*, 2011). Within the PDZ1 the arginine residue at position 499 (K499) was shown to be crucial for the interaction with E6 PBM, and its mutation to glutamic acid (K499E) strongly reduces the strength of the interaction (Fournane *et al.*, 2011). However, what the functional consequences are of the disruption of the interaction between MAGI-1 and E6 in context of the HPV pathology, remains an open question.

Targeting of PDZ-dependent functions not associated with cell junctions

Although high-risk HPV E6 targets a number of PDZ domain-containing proteins associated with junctional complexes, it has also been shown to interact with PDZ-proteins associated with other activities, and a list of the identified E6 PDZ substrates is shown in Table 1. These include the protein phosphatases PTPN3 and FAP1, the Tax-interacting proteins (TIP)-1 and -2 and CFTR (cystic fibrosis transmembrane regulator)-associated ligand (CAL) (reviewed in Thomas *et al.*, 2008b).

TIP-1 and TIP-2 were originally discovered as HTLV-1 Tax-binding proteins in a yeast two-hybrid screen for PDZ-containing binding partners for Tax-1 (Rousset *et al.*, 1998), and subsequently both have been shown also to interact with high-risk E6 oncoproteins (Hampson *et al.*, 2004; Favre-Bonvin *et al.*, 2005). TIP-2 interacts with the GTPase-activating protein (GAP) GAIP, which associating with Gα subunits of heterotrimeric G proteins, promotes their rapid cycling from GTP- to GDP-bound state, thereby accelerating their inactivation (De Vries *et al.*, 1998). One of the functions associated with TIP-2 expression is the modulation of cell responsiveness to TGFβ, by driving the expression of type III TGFβ receptors at the cell surface through the direct interaction with its class I PBM. This results in the activation of TGFβ-responsive genes and inhibition of cell

growth (Blobe *et al.*, 2001). HPV-18 E6 has been shown to interact with and drive the proteasome-mediated degradation of TIP-2 and, consistent with its biological function, loss of TIP-2 correlated with a decrease in TGFβ-mediated cell growth inhibition of HPV-positive cells (Favre-Bonvin *et al.*, 2005). In marked contrast with other PDZ-containing proteins, TIP-1 activity was shown to be promoted by the presence of HPV-16 E6. Structurally the TIP-1 protein is composed almost

Table 1. Identified PDZ-containing targets for viral oncoproteins.

Target PDZ protein	Targeting viral oncoprotein	Effects on target protein	Target protein function	References
hDlg	HPV E6	Degradation/sequestration	Cell polarity / context dependent function (?)	Gardiol *et al.*, 1999
	Ad9 E4-ORF1	Sequestration		Lee *et al.*, 1997
	HTLV-1 Tax1	Sequestration		Hirata *et al.*, 2004
hScrib	HPV E6	Degradation	Cell polarity / tumor suppressor	Nakagawa and Huibregtse, 2000
	HTLV-1 Tax1	Sequestration		Okajima *et al.*, 2008
MAGI-1	HPV E6	Degradation,	Cell polarity / tumor suppressors	Glaunsinger *et al.*, 2000
	HTLV-1 Tax1	Sequestration		Makokha *et*

				al., 2013
	Ad9 E4-ORF1	Sequestration		Glaunsinger *et al.*, 2000
MAGI-2	HPV E6	Degradation		Thomas *et al.*, 2002
MAGI-3	HPV E6	Degradation		Thomas *et al.*, 2002
	HTLV-1 Tax1	Sequestration		Ohashi *et al.*, 2004
MUPP1	HPV E6	Degradation	Cell polarity	Lee *et al.*, 2000
	Ad9 E4-ORF1	Sequestration		Lee *et al.*, 2000
PATJ	HPV E6	Degradation,	Cell polarity	Latorre *et al.*, 2005; Storrs ans Silverstein, 2007
	Ad9 E4-ORF1	Sequestration		Latorre *et al.*, 2005
PSD95	HPV E6	Degradation	Cell polarity / tumor suppressor	Handa *et al.*, 2007
TIP-1	HPV E6	Stabilization	p53 inactivation	Hampson *et*

			/ inhibitor of PDZ-mediated interactions	*al.*, 2004
TIP-2	HPV E6	Degradation	Modulator of TGFβ signalling	Favre-Bonvin *et al.*, 2005
PTPN3/PTPH1	HPV E6	Degradation	Modulation of p38γ / context-dependent function	Jing *et al.*, 2007; Töpffer *et al.*, 2007
FAP1/PTPN13	HPV E6	Degradation	Modulation of Fas-mediated apoptosis / context-dependent function	Spanos *et al.*, 2008b
CAL	HPV E6	Degradation	CFTR trafficking and degradation	Jeong *et al.*, 2007
ZO-2	Ad9 E4-ORF1	Sequestration	Cell polarity/tumor suppressor	Glaunsinger *et al.*, 2001

exclusively of a single PDZ domain, and it has been proposed to antagonize PDZ-mediated interactions by competing with other PDZ-proteins for the binding to target PBMs (Alewine *et al.*,

2006). A pro-oncogenic function for TIP-1 has been proposed, with the activation of Rho-dependent gene expression, exerted through the interaction with the PBM of the Rho effector rhotekin (Reynaud *et al.*, 2000). Consistent with the latter studies, expression of TIP-1 in HPV-16 E6-expressing cells has been shown to be required to activate RhoA signaling (Hampson *et al.*, 2004).

A contribution to the stimulatory effect of cell growth has been suggested to be given by E6 through the targeting of PDZ domain-containing cellular phosphatases. Three PDZ domain-containing protein tyrosine phosphatases (PTPs), FAP-1 (PTPN13), PTPH1 (PTPN3) and PTPMEG1 (Alonso *et al.*, 2004), have been described, with PTPH1 and FAP-1 closely linked to tumorigenesis. Although their roles in tumorigenesis are still controversial, FAP-1 and PTPH1 have been shown to be inactivated by high-risk HPV E6 oncoproteins, supporting a tumor-suppressor function in the context of the HPV pathology. Early studies suggested a possible tumor-suppressor potential for PTPH1, since mutations were reported in some colorectal cancers (Wang *et al.*, 2004): however, more recent studies are indicative of pro-oncogenic activity. K-Ras was shown to increase the expression levels of PTPH1 and p38γ (Hou *et al.*, 2010), with PTPH1 and p38γ interacting in a PDZ–PBM-dependent manner. This results in dephosphorylation of phospho-p38γ and inhibition of the phospho-p38γ-mediated down-regulation of Ras signalling (Han and Sun 2007; Hou *et al.*, 2010), thereby generating a positive-feedback loop. PTPH1 was also found to be overexpressed in a number of breast cancers, resulting in a perturbation of vitamin D receptor localization and stimulation of cell proliferation (Zhi *et al.*, 2011). PTPH1 has been recently identified as proteolytic substrate for HPV-16 E6 oncoprotein in human keratinocytes (Jing *et al.*, 2007; Töpffer *et al.*, 2007). Expression of HPV-16 E6 in human immortalized keratinocytes correlated with the acquisition of cell growth capabilities with reduced nutrient requirements, in a PBM-dependent manner. Silencing of PTPH1 partially reproduced the ability of keratinocytes to grow under stringent conditions, suggesting a contribution of loss of PTPH1 toward this phenotype (Jing *et al.*, 2007). Although FAP-1 has been reported to be regulated through its localization at AJs, it possesses a broader spectrum of biological activities. FAP-1 has been shown to be a potent negative regulator of the Src signaling, and a regulator of the Fas-

mediated apoptotic pathway (Cuppen *et al.*, 2000; Ungefroren *et al.*, 2001; Lai *et al.*, 2007; Glondu-Lassis *et al.*, 2010). Recently, HPV-16 E6, but not its PBM-deletion mutant, has been reported to promote anchorage-independent growth in tonsil epithelial cells and invasive cell growth in cooperation with activated Ras (Spanos *et al.*, 2008a, Spanos *et al.*, 2008b). Likewise, loss of FAP-1 was sufficient to promote anchorage-independent growth of epithelial cells, and depletion of the phosphatase cooperated with Ras to promote invasiveness (Spanos *et al.*, 2008b). This suggest that these oncogenic activities of E6 are mediated, at least in part, through the inactivation of FAP-1.

CAL is a PDZ-containing protein associated with the Golgi apparatus. It is involved in the intracellular trafficking of different receptors (Yao *et al.*, 2001; Hassel *et al.*, 2003; Gentzsch *et al.*, 2003), and it was shown to promote the lysosomal degradation of CFTR, hence reducing its exposure on the cell surface (Cheng *et al.*, 2002; Cheng *et al.*, 2004). Recently, CAL was described as a novel binding partner for HPV-16 E6, and is targeted for proteasome-mediated degradation by HPV-16 and -18 E6 oncoproteins, although it is a preferential substrate for 16 E6 (Jeong *et al.*, 2007). Interestingly, CFTR expression was described as a prognostic marker for malignant progression of cervical cancer (Peng *et al.*, 2012), hence the inactivation of CAL by E6 could significantly contribute to cervical cancer progression through the elevation of CFTR levels.

HPV life cycle

Papillomaviruses (PVs) are a broad group of small unenveloped DNA viruses, infecting a wide variety of vertebrates. A recent classification, listed 189 PV types in the Papillomaviridae family, organized in 29 genera according to their genetic and pathological characteristics. Of these, 5 genera include the HPVs (Bernard *et al.*, 2010). This heterogeneity is reflected by different replicative modalities, due to differences in their gene products, sites of infection, patterns of interaction with host factors and responsiveness to external stimuli. The best studied group are the alpha papillomaviruses, which infect cutaneous and mucosal epithelia with clinical manifestations ranging from self-remissive infections associated with benign warts to persistent infections that can eventually lead to anogenital and head and neck cancer. Within the alpha PV types, 12 HPV types

have been defined as cancer-causing, with HPV-16 and -18 being predominant, accounting for about 70% of the global cervical cancer cases (zur Hausen, 2002), of which there are over 500 000 new cases every year (Forman *et al.*, 2012).

The HPV life cycle is intimately linked to the differentiation program of the infected keratinocytes, where the expression of specific viral gene products and maturation of viral particles coincides with the induction of differentiation in the infected cells. Figure 7 shows the genomic organization of HPV-16 and the sequential activation of the HPV genome in the infected epithelium. In the generally accepted model for HPV infection, viral particles are thought to penetrate stratified epithelia through micro-lesions, resulting in the localization of the virus at the basal lamina where it can infect stem cells or transiently amplifying cells of the basal epithelial layer, the natural host cells of HPV (Stubenrauch and Laimins, 1999; Pyeon *et al.*, 2009). The pro-inflammatory response evoked during wound-healing processes, as well as the transient elevation of growth factors at the site of infection in the basal layer, are also thought to be beneficial for successful infection through the stimulation of cell proliferation. Recent evidence suggests that the mitotic progression through the cell-cycle and nuclear membrane break-down are also required for successful viral infection, allowing nuclear entry of the viral DNA (Peyon *et al.*, 2009). In stratified epithelia only the cells in the basal layer are mitotically active, and this might in part provide an explanation for the tropism of HPV for these cells. At initial stages of infection, viral gene expression is activated resulting in the production of 20 to 100 copies of episomal viral DNA. Episomes are stably maintained in basal layer cells (Sterling *et al.*, 1990; De Geest *et al.*, 1993), and induction of differentiation results in the viral DNA vegetative amplification in the suprabasal epithelial layers, where viral DNA is amplified to a high copy number and ultimately packaged in the new virions. These three phases of the viral life cycle are accomplished by the activation and sequential expression of "early" and "late" viral proteins within the differentiating epithelium.

As mentioned above, the primary function of DNA tumor virus-encoded oncoproteins is to provide an environment that can support the viral life cycle. The two major HPV oncoproteins are E6 and E7, although E5 also displays important oncogenic functions (see below). The expression of HPV-

16 E6 and E7 from the early region of the viral genome is under the control of the p97 promoter and begins at early stages after the virus entry. The expression of E6 and E7 in the basal layer clonally expands the population of infected cells that are competent to progress to the differentiation-promoted stages of viral life cycle (i.e. expression of late transcripts and release of viral particles). In stratified epithelia, the basal layer represents the reservoir of actively proliferating cells. As basal cells divide, a population of daughter cells is pushed to migrate in the suprabasal layers whilst the basal cell population is maintained as slow-cycling and self-renewing cells. In virus-free epithelia, cells that leave the basal layer exit the cell cycle, cease to replicate their DNA and, as a result of the terminal differentiation program, they lose their nuclei in the upper layers of the epithelium. Therefore, HPV needs to reprogram this process and force the cell cycle entry of differentiating cells in order to replicate the viral DNA and establish the infection; this is accomplished mainly through the expression of E6 and E7, which are expressed from initial phases of infection onwards. As mentioned above, E7 oncoproteins play an active role in the induction of cell proliferation through the inactivation of members of the pRB family, whilst E6 prevents the activation of apoptotic pathways induced by E7, through the inactivation of p53. The cell-cycle entry in the basal cell layer is mainly controlled by pRB and p107, whereas the S-phase re-entry in differentiating cells is preferentially regulated by p130, likely reflecting the availability of different E2F family members of transcription factors during different phases of epithelial differentiation (Paramio *et al.*, 1998; Litovchick *et al.*, 2004). Thus, E7 proteins derived from high-risk HPV types through the inactivation of all the members of the pRB protein family, drive the acquisition of a S-phase competent phenotype throughout the epithelium (Zhang *et al.*, 2006; Zerfass *et al.*, 1995a). This also highlights the molecular basis for the different pattern of cell-cycle activation seen in the pathology linked to low-risk HPV infection, where S-phase competent cells are present primarily in the suprabasal layers of the epithelium (Doorbar *et al.*, 2012). In addition, the CKII-mediated phosphorylation of E7 increases the ability of E7 to bind and inactivate p130 (Genovese *et al.*, 2008), suggesting that changes in the levels of CKII phosphorylation of E7 might affect the ability of E7 to perturb pocket protein function. Furthermore, the ability of E7 to promote the activation of E2F responsive genes depends on its ability to impair the function of cellular

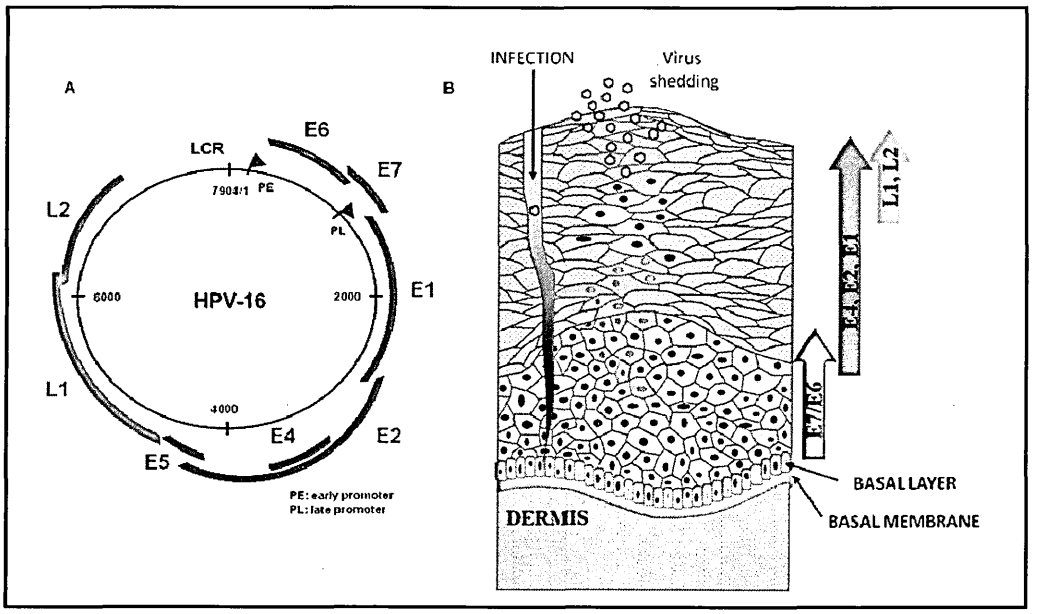

Figure 7. Schematic representation of HPV-16 genome and the sequential expression of viral gene products during differentiation of the infected epithelium. A. The ORFs encoding the different viral genes are positioned along the genome. The position of the early and late promoters, as well as of the long control region (LCR) are also depicted. B. The figure shows the presumed route of HPV infection of the epithelial mucosa basal cell layer through microtraumas. There is coordinate expression of the different viral gene products in a differentiation-regulated manner, with E6/E7 causing an expansion of S-phase competent cells. This allows viral genome amplification and, ultimately, the synthesis and shedding of new viral particles within a period of 2–3 weeks (adapted from Middleton *et al.*, 2003).

histone deacetylases (HDACs) (Brehm *et al.*, 1999; Longworth *et al.*, 2005), and the activation of E2F-responsive promoters by E7 is dependent upon the integrity of both its pRB and HDAC binding sites (Zhang *et al.*, 2004; Zhang *et al.*, 2006). The ability of E7 to promote S-phase entry, is also aided by its interaction with cyclin-dependent kinase (CDK) inhibitors $p21^{cip}$ and $p27^{kip1}$, leading to their inactivation (Zerfass-Thome *et al.*, 1996; Funk *et al.*, 1997; Jones *et al.*, 1997b) and upregulation of the S-phase cyclins A and E (Zerfass *et al.*, 1995b). The inhibitory effect of $p21^{cip}$ and $p27^{kip1}$ are in part driven by E7, also through the up-regulation of Akt activity. Akt, or PKB, is a serine/threonine kinase activated upon the sequential phosphorylation by PDK1 and mammalian target of rapamycin complex 2 (mTORC2), which are downstream targets of the growth factor-responsive phosphoinositide 3-kinase (PI3K). Akt regulates several proto-oncogenic processes, including proliferation, cell survival and protein translation, the last of which is mediated through the regulation of the "nutrient sensor" mTORC1 and its associated signaling (Ma and Blenis, 2009). In the stratified epithelium the activity of proto-oncogenic pathways, such as Ras and PI3K, are restricted in the basal layer and are lost during differentiation (Dajee *et al.*, 2002; Menges *et al.*, 2006). The disruption of pRB-E2F complexes by viral oncoproteins has been suggested to promote the stimulation of PI3K and Akt, through the activation of the E2F-responsive gene Gab2, a positive mediator of PI3K signaling (Chaussepied and Ginsberg, 2004). The HPV-16 E7-mediated activation of Akt has been shown to promote the cytoplasmic retention of the CDK inhibitors $p21^{cip}$ and $p27^{kip1}$ upon oncogenic insult (Westbrook *et al.*, 2002; Charette and McCance, 2007). The E7-induced mislocalisation of p21 and p27 correlated respectively with loss of oncogene-induced cell cycle arrest and increased migration of human foreskin keratinocytes (HFKs), and both these effects could be reverted upon inhibition of Akt (Westbrook *et al.*, 2002; Charette and McCance, 2007). Interestingly, low-risk E7 oncoproteins are also able to stimulate the activity of Akt, however this occurs through a pocket protein-independent mechanism (Pim *et al.*, 2005). Thus, E7 induces a pseudo S-phase condition in parabasal layers of the epithelium, establishing an environment suitable for the viral genome amplification.

As mentioned above, in normal epithelia, the expression of $p14^{ARF}$ and activation of p53 can function as a molecular sensor of unscheduled DNA replication. However, the expression of E6

prevents the activation of these pathways by promoting the proteasome-mediated degradation of both hAda3 and p53 itself, an activity dependent upon the host E3 ubiquitin-ligase E6AP (Scheffner *et al.*, 1990; Scheffner *et al.*, 1993; Shamanin *et al.*, 2008; Hu *et al.*, 2009). Conversely, low-risk E6 proteins are thought to perturb p53 function by relieving its repression on TATA-containing promoters (Lechner *et al.*, 1992) and hindering the activation of p53-responsive genes (Mietz *et al.*, 1992; Pim *et al.*, 1994). Moreover, it was shown that E6-interacting regions of p300 are necessary for E6 to inhibit p53-dependent transcription and that E6 activity can inhibit the acetylation of both p53 and nucleosomal core histones, without altering p53 and p300 recruitment to chromatin. This process is E6AP-independent and shows a unique mechanism of E6 repression of p53 activity which does not involve proteasomal degradation (Thomas and Chiang, 2005). In line with a central role for p300/CBP in the life cycle of many different viruses, several other DNA tumor viruses have also been shown to interact with p300/CBP (Goodman and Smolik, 2000; reviewed in Moore and Chang, 2010), highlighting its central role in regulating cellular homeostasis. As for other E6 targets, high-risk HPV E6 oncoproteins were reported to bind strongly to p300/CBP whereas the association with low-risk HPV-derived E6 is weaker (Patel *et al.*, 1999). It was also shown that HPV-16 E6 inhibits the intrinsic transcriptional activity of p300/CBP on both p53 and NFκB-responsive promoter elements. In the case of p53 this is partly due to an inhibition of p300-mediated acetylation (Thomas and Chiang, 2005).

High-risk, but not low-risk, E6 proteins are able to reactivate the telomerase complex, partly in cooperation with E7, thereby contributing towards prolonging the lifespan of infected cells (Klingelhutz *et al.*, 1994; Klingelhutz 1996; DeFilippis *et al.*, 2003; Xuefeng *et al.*, 2009). An interesting function of E6, shared with E7, is its ability to activate PI3K/Akt signaling. Expression of HPV-16 E6 in primary keratinocytes, has been found to be sufficient to sustain the PI3K signaling under conditions of nutrient deprivation, through the aberrant hyper-activation of growth factor receptors (Spangle and Münger., 2013). This activity of E6 correlated with the stimulation of mTORC1 and increased cap-dependent protein synthesis (Spangle and Münger, 2010). Interestingly, the effect produced by high-risk E6 oncoproteins on protein translation was shown to be dependent upon its interaction with the E6AP and, in part, on its PBM. Consistent with this,

low-risk HPV E6 oncoproteins, which are able to interact with E6AP but lack the PBM, were able to activate protein translation, albeit to a lesser extent compared with HPV-16 and -18 E6 oncoproteins (Spangle and Münger, 2012).

An important factor in the establishment of infection, is the modulation of the host immune system response. This evasion can lead to the establishment of persistent infections, the prime risk factor for malignant progression (Bodily and Laimins, 2011). HPV exerts a global effect on the innate immune response in infected keratinocytes; pro-inflammatory cytokines, including type-1 interferon, are not released and signals required for the activation and migration of intraepithelial Langerhans cells (LCs) and macrophages are inadequate (Kanodia *et al.*, 2007). High-risk HPV E6 and E7 actively contribute to this through the deregulation of the innate immune response. They cooperate to down-regulate the expression of interferon (INF)-responsive genes, including INF-α and -β (Nees *et al.*, 2001). The two oncoproteins also perturb the signaling pathways activated following INF stimulation; E6 interferes with the activation of Jak-STAT signaling upon INF-α treatment (Li *et al.*, 1999), whereas E7 blocks the activity of interferon responsive element (IRF)-1 and NFkappaB following exposure of cells to interferon stimulation (Perea *et al.*, 2000; Um *et al.*, 2002). In addition, perturbation of cell polarity by E6 and E7 through the reduction of cell surface E-cadherin exposure contributes to impair the recruitment of LCs to infected epithelial cells, ultimately leading to the depletion of LCs from the infected epithelium and inadequate T-cell priming (Matthews *et al.*, 2003). Recently, PDZ proteins have also been shown to contribute directly to the regulation of the interferon response (Werme *et al.*, 2008; Kumar *et al.*, 2012), suggesting that E6's PDZ-binding activity could be relevant for the modulation of innate immunity, although the experimental evidence is still missing.

The viral E1 and E2 proteins are among the first viral proteins to be expressed in the infected epithelium (Longworth and Laimins, 2004b). E2 is a DNA-binding protein that recognizes multiple binding sites in the LCR (long control region) of the viral genome. The association of E2 with E1 recruits the latter to viral origins, where the recruitment of cellular polymerases begins the viral DNA replication (Sedman *et al.*, 1997; Dixon *et al.*, 2000). E1 possesses a helicase activity but has

a weak DNA binding activity, and it requires the interaction with E2 to mediate its association with viral origins (Sedman *et al.*, 1997; Dixon *et al.*, 2000). In addition, studies with the bovine papillomavirus type 1 (BPV1), revealed that E2 plays an important function in tethering the viral episomal DNA to host chromosomes, thereby ensuring an equal partitioning of replicated viral DNA to daughter cells (Skiadopoulos and McBride, 1998; Ilves *et al.*, 2006). In BPV1, the host bromodomain protein Brd4 mediates the association of E2 with chromatin (You *et al.*, 2004; McPhillips *et al.*, 2006) and stimulates the E2-dependent transcription activation (Ilves *et al.*, 2006). Conversely, HPVs rely on different cellular factors in order to mediate the tethering of viral DNA to chromatin through E2 (Parish *et al.*, 2006), although Brd4 is required for the E2-mediated viral DNA transcription (McPhillips *et al.*, 2006). It is important to note that intact E1 and E2 ORFs appear to be required for the stable maintenance of BPV1 viral episomes, (Sarver *et al.*, 1984), and that the DNA-binding activity of E2 prevents the integration of HPV-31 genome into the host (Stubenrauch *et al.*, 1998a). Viral genome integration leads to unscheduled E6 and E7 expression, and has been proposed as one of the events leading to malignant progression of HPV infections (Jeon *et al.*, 1995b; Song *et al.*, 2000). Moreover, binding of E2 to its DNA-binding sites in the LCR has been shown to repress the activity of the early promoter (p97) (Steger and Corbach, 1997; Stubenrauch *et al.*, 1998a). Thus, this ability allows E2 to have an indirect inhibitory effect on cell proliferation through the repression of E6 and E7 expression (Francis *et al.*, 2000).

The E5 protein is highly hydrophobic and interacts with the 16-kD pore-forming protein component of the vacuolar H^+-ATPase, responsible for acidifying cellular organelles such as the Golgi apparatus (Conrad *et al.*, 1993). E5-mutant HPV-16 and -31 genomes exhibit reduced DNA synthesis in the suprabasal layers of infected epithelia (Genther *et al.*, 2003; Fehrmann *et al.*, 2003a) and hence impaired expression of late viral proteins (Fehrmann *et al.*, 2003), however E5's role in HPV genome amplification is controversial. One of the most prominent functions of E5 is its ability to potentiate EGF signaling and drive the activation of MAP kinase pathways (Conrad *et al.*, 1994; Crusius *et al.*, 1998; Crusius *et al.*, 2000). Importantly, ERK kinases 1 and 2 have been show to be critical modulators of the nuclear accumulation of the viral helicase E1, where

phosphorylation by cyclin A and B/Cdk2 complexes inhibit the shuttling of E1 to the cytoplasm (Deng *et al.*, 2004; Yu *et al.*, 2007). However, loss of E5 function in the context of HPV-16 and -31 infection only marginally affected the viral genome amplification (Genther *et al.*, 2003; Fehrmann *et al.*, 2003), suggesting that host and viral DNA synthesis are uncoupled. The modulation of host mitogenic pathways, along with the ability to inhibit apoptosis (Kabsch *et al.*, 2004), supported a possible role for E5 in cancer progression together with E6 and E7. The expression of E5 induces the potentiation of the epidermal growth factor (EGF)-mediated mitogenic signal in multiple cell lines (Pim *et al.*, 1992; Leechanachai *et al.*, 1992) and cooperates with E7 to transform primary rodent epithelial cells (Bouvard *et al.*, 1994; Valle and Banks, 1995). Nevertheless, during cancerous progression in most cases large parts of the viral genome, including the E5 ORF are deleted upon integration of the viral DNA into the host genome (Schwarz *et al.*, 1985), although the potentiation of EGFR signalling by E6 might compensate for loss of E5 (Akerman *et al.*, 2001).

The expression of E4 is under the control of the differentiation-dependent promoter, and it is the most highly expressed of the viral proteins. Its accumulation is dependent upon the formation of amyloid-like insoluble structures driven by the C-termini of E4 proteins (McIntosh *et al.*, 2008). High level of E4 expression is a surrogate marker for efficient viral replication (Doorbar *et al.*, 1997), associated with productive viral life cycle and low grade lesions (Doorbar *et al.*, 2012), whereas its expression is progressively lost during malignant progression. A role for E4 in mature virion release has been proposed, since E4 is able to interact with and reorganize cytokeratins (Doorbar *et al.*, 1991; Wang *et al.*, 2004). Loss of the integrity of the keratin network is thought to favor the viral egress (Doorbar *et al.*, 1991). In addition, E4 promotes the G2 arrest of infected cells through the modulation of cyclin/Cdk complexes (Knight *et al.*, 2004; Davy *et al.*, 2005; Knight *et al.*, 2006; Davy *et al.*, 2006). This activity is thought to be important for uncoupling viral genome replication from mitosis, thus supporting HPV genome amplification while blocking progression through the cell cycle. Evidence for the relevance of E4 functions in context of the viral life cycle come from studies where loss of E4 reduced vegetative replication and expression of late

transcripts, ultimately leading to reduced virion formation (Peh *et al.*, 2004; Nakahara *et al.*, 2005; Wilson *et al.*, 2005).

The viral capsid proteins L1 and L2 are expressed in the late stages of infection, after viral genome amplification. While both capsid proteins are essential for the assembly of infectious viral capsids (Zhou *et al.*, 1993; Holmgren *et al.*, 2005; Kämper *et al.*, 2005), L1, the major viral capsid protein, can spontaneously assemble into VLPs (Kirnbauer *et al.*, 1992), and this ability of L1 provides the basis for the HPV vaccine (Kirnbauer *et al.*, 1992). Conversely, L2, the minor capsid protein, regulates the early stages of infection, promoting the shuttling of viral DNA from late endosome/lysosome compartments to the host cell nucleus. In the later stages of infection, it enhances the viral genome encapsidation, ensuring the successful completion of the productive stage of the life cycle (Holmgren *et al.*, 2005).

From productive viral life cycle to cancer and the significance of PDZ-protein targeting

An important feature of HPV and other tumor virus life cycles, is their ability to establish long-term persistent infection in their hosts (Banks *et al.*, 2012). In order to persist, HPV needs to evade the host immune response. This is achieved in part by the E6 and E7 through the modulation of the innate immune response, but is also aided by the peculiarities of the HPV life cycle; it is exclusively intraepithelial, there is no viraemia and it is not associated with cytolysis or inflammation (Stanley, 2012b). The steps believed to be involved in the progression from productive viral life cycle to cervical cancer are depicted in Figure 8. Nevertheless, the vast majority of HPV infections (80-90%) result in low-grade lesions, and, as suggested by animal models of HPV infection, are cleared as a result of the generation of a CD4 and CD8+ T cell-mediated immunity which leads to the regression of the lesion (Nicholls *et al.*, 2001; Monnier-Benoit *et al.*, 2006). However, the lack of an effective cell-mediated immune response able to control the infection can lead to persistent infection and, in the case of cancer-causing HPV types, to an increased risk of developing high-grade lesions and invasive carcinoma. Long term infections imply that the cells have to support the expression of viral oncoproteins for long periods of time, thus increasing the risk of accumulating proto-oncogenic mutations and the likelihood of

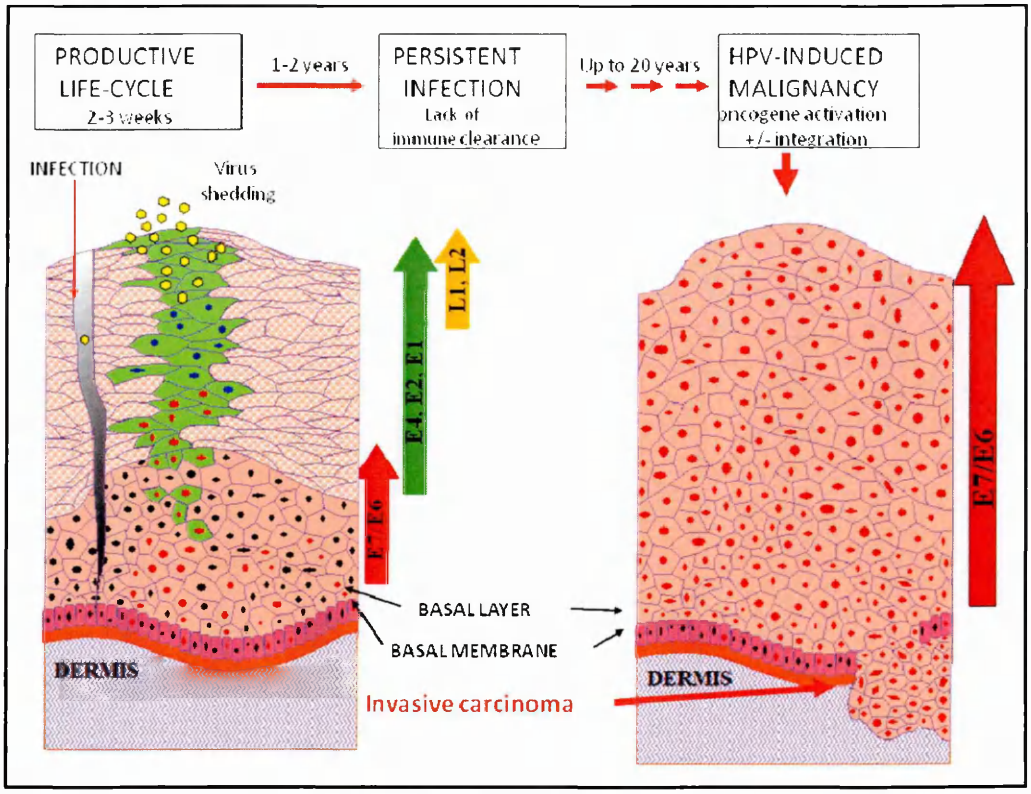

Figure 8. Schematic representation of the events believed to be involved in the progression from productive HPV infection (left-hand panel) to malignancy (right-hand panel). Upon HPV infection, the lack of immune clearance might lead to a persistent infection in which lesions are not resolved and viral DNA can be detected over extended periods of time. This ultimately predisposes the host to the development of a malignancy. This is characterized by a loss of differentiation, no viral replication and high levels of E6 and E7 oncoprotein expression (adapted from Middleton *et al.*, 2003).

"mistakes" that eventually might lead to deregulation of the viral life cycle and/or viral DNA integration into the host genome. In HPV-infected epithelia, the viral life cycle can become deregulated as the result of the perturbation of viral gene expression, frequently leading to viral genome integration and progression to malignancy (Doorbar *et al.*, 2012). This model appears to be confirmed by the observation that epithelia that are not permissive for the viral life cycle are sites where infection by high-risk HPV types can progress very rapidly to cancer, with high rates of viral genome integration (Herfs *et al.*, 2012). In epithelia permissive for the viral life cycle, such as columnar and squamous cervical epithelia, where most of the research has been carried out, productive infection is primarily associated with low grade lesions, or cervical intraepithelial neoplasia grade 1 (CIN1), where tightly controlled expression of E6 and E7 sustains all the phases of productive viral life cycle. In this context, the consequences of deregulation of PDZ-containing proteins by high-risk E6 oncoproteins is still poorly understood. In stratified epithelia, symmetric divisions of basal cells occur parallel to the basal membrane axis, producing two daughter cells phenotypically identical that preserve the ability to proliferate and self-renew. Conversely, asymmetric divisions, perpendicular to the basal membrane produce a basal stem cell and an apical cell that is committed to differentiate (Muroyama and Lechler, 2012), involving the reorientation of the mitotic spindle and unequal distribution of cell fate determinants between daughter cells (Lechler and Fuchs, 2005). Although the mechanism that controls the switch from symmetric to asymmetric division in basal cells is poorly understood, compelling evidence suggests that reorientation of mitotic spindles requires components of the apico-basal polarity control (Roegiers and Jan, 2004). Therefore, HPV is potentially able to subvert the control of spindle orientation through E7's stimulation of the cell cycle combined with the E6 perturbation of hScrib, hDlg and components of apical polarity complexes, all of which have been shown to direct the spindle orientation in epithelial cells (Knoblich, 2008; Johnson *et al.*, 2009; Hao, 2010). This could contribute towards increasing the population of infected basal cells able to sustain viral replication and expand the area of the lesion by lateral spreading of infected cells. Although the status of most of the HPV PDZ-containing targets is still obscure in context of the viral life cycle, hDlg localization was found to be aberrantly cytoplasmic in the suprabasal layers of CIN1 lesions

(Cavatorta *et al.*, 2004). This was similar to the pattern of hDlg distribution in the basal epithelial layers of the cervix, suggesting that HPV infection delays differentiation of epithelial tissues. Perturbation of PDZ polarity proteins by E6 in the suprabasal layers is likely to perturb the assembly of TJs, which normally takes place in the differentiated granular layer (Brandner *et al.*, 2002; Kirschner and Brandner, 2012). As discussed previously, assembly of TJs promotes the formation of segregated microenvironments in the cell that prevents the generation of proliferative cues. Thus, after establishment of HPV infection in wounded epithelia, the targeting of PDZ proteins and other components of the epithelial polarity machinery by HPV oncoproteins might delay the restoration of intact junctional complexes and of the epithelial barrier function. Ultimately, this would prolong the exposure of infected cells to proliferative cues, favoring the expansion of the HPV-positive cell population. A summary of the processes potentially targeted by HPV through the inactivation of PDZ domain-containing proteins is depicted in Figure 9. High-risk HPV genomes can be efficiently maintained as episomes in human keratinocytes, and episomal maintenance is dependent upon the functions of E6 and E7 and also of the viral replicative proteins E1 and E2 (reviewed in Doorbar *et al.*, 2012). Human foreskin keratinocytes (HFKs) expressing wild type HPV-31 genomes display marked hyperplasia in suprabasal layers when cultured in organotypic raft cultures (Lee and Laimins, 2004). This effect was associated with the ability of HPV-31 E6 to interact with PDZ-containing proteins since, mutant genomes expressing a PDZ-defective E6 failed to promote the hyperplastic phenotype. HPV-31 genomes, where the E6 PBM has been mutated, provide evidence that loss of PDZ-binding activity results in the abolition of the replicative viral life cycle by the increased tendency of viral DNA to integrate into the host genome (Lee and Laimins, 2004). Recently, similar results have been obtained also with HPV-16 genomes stably expressed in immortal human keraticocytes (NIKS) (Nicolaides *et al.*, 2011). In this study, genomes expressing a truncated form of E6, lacking its PBM, had an increased propensity to integrate in the host genome. However, this correlated with a reduced stability of PBM-defective E6 oncoproteins (Nicolaides *et al.*, 2011).

Although the mechanism is still unknown, these studies suggest that the ability of E6 to interact with PDZ domain-containing proteins is important for the establishment of viral copy numbers in

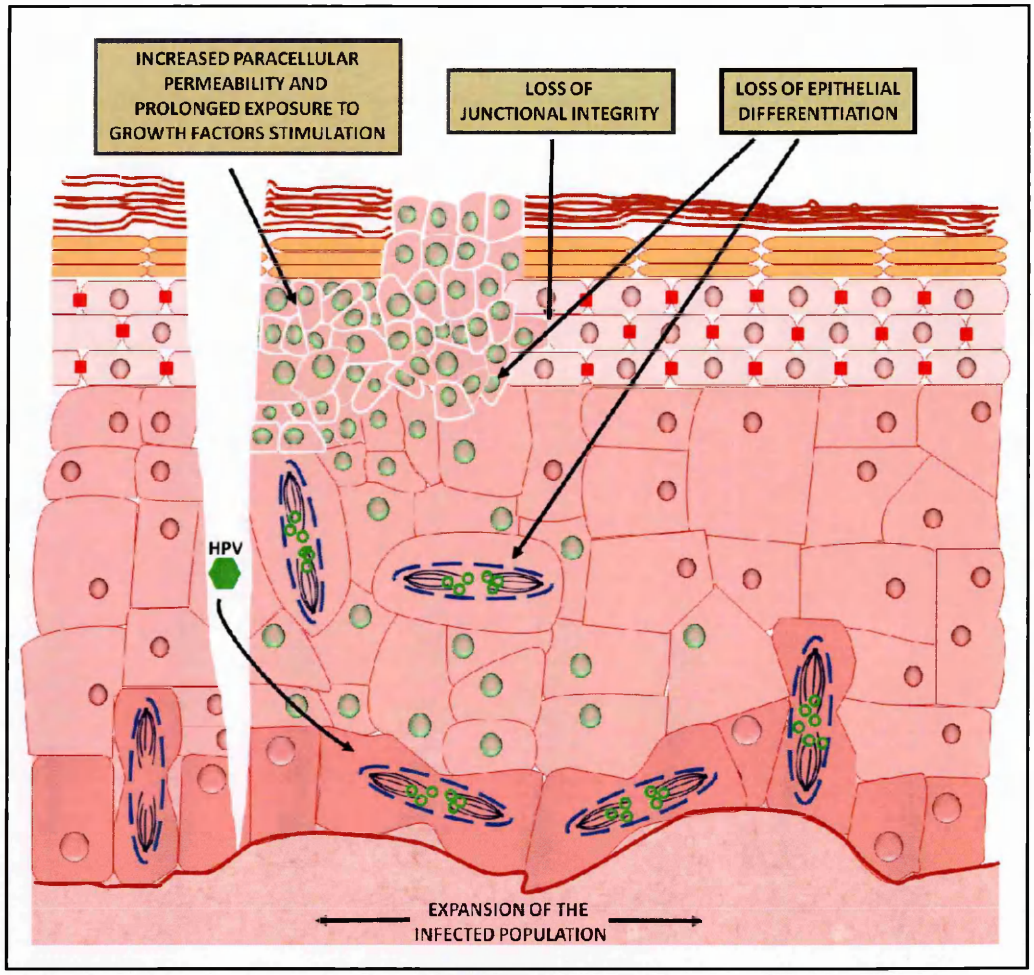

Figure 9. Possible roles for the targeting of PDZ domain-containing proteins in the HPV pathology. Following infection of basal cells, HPV genomes are maintained as episomes (green circles in dividing cells). The expression of HPV oncoproteins in these cells might lead to the expansion of the basal cell population harboring HPV genomes. In this scenario, the targeting of PDZ domain-containing proteins by E6 might contribute through the alteration of the normal wound healing response and defects in the mitotic spindle orientation due to loss of cell polarity. In the suprabasal layers, targeting of PDZ proteins might interfere with the normal differentiation process by preventing the formation of junctional complexes. Furthermore, this could also increase the exposure of infected cells to mitogenic stimuli, further supporting viral genome amplification and the vegetative life cycle.

basal layers of infected epithelia, and is essential for the correct progression of productive life cycle. Although a subset of HPV-16-positive cervical cancers can arise from cells containing exclusively episomes (Matsukura *et al.*, 1989; Pett and Coleman, 2007; Vinokurova *et al.*, 2008), deregulation of the viral life cycle due to viral DNA integration, has been proposed to be a hallmark of malignant progression of HPV infection (Cullen *et al.*, 1991; Pirami *et al.*, 1997; Badaracco *et al.*, 2002; Woodman *et al.*, 2003), and is associated with transition from low grade productive lesions to higher grade lesions (CIN3), where the ability of the virus to replicate is progressively lost. This condition is believed to be associated with the elevation of E6/E7 expression, predisposing infected cells to accumulate mutations and leading to the development of overt cancer.

HPV and cancer

It is currently estimated that about 20% of cancer cases worldwide are caused by an infectious agent. It is important to note that infections leading to cancer in humans can have both viral and non-viral origins, and that 70% of these (about 15% of total cancer cases) are nonetheless linked to viruses. In terms of prevalence in human cancers, however, HPV-associated cancers are the most abundant, with HPV infection being responsible for approximately 5% of the cancer burden worldwide (Parkin and Bray, 2006).

Experiments in tissue culture systems and animal models showed the importance of the combined E6 and E7 activities for cellular immortalization and transformation (Hawley-Nelson *et al.*, 1989; Matlashewski *et al.*, 1987; Riley *et al.*, 2003). Furthermore, in HPV-positive cells the inhibition of E6 and E7 expression, by re-expression of the HPV transcriptional repressor E2, or by siRNA ablation, results in the inhibition of cell growth and induction of cell death by apoptosis, thus highlighting the strict requirement of HPV-transformed cells for the continuous expression of E6 and E7 (Alvarez-Salas *et al.*, 1998; Yoshinouchi *et al.*, 2003). It is important to point out, however, that in primary keratinocytes, the natural target of HPV infection, E6 displays only weak transforming capacity in absence of E7, and that both oncoproteins are required to promote cell immortalization (Barbosa and Schlegel, 1989; Hawley-Nelson *et al.*, 1989; Münger *et al.*, 1989;

Watanabe *et al.*, 1989). An interesting feature of HPV oncoproteins deriving from these assays is that only E6 and E7 oncoproteins deriving from high-risk HPV types drive immortalization, whereas those expressed by low-risk types have weak or no immortalizing capacity (Schlegel *et al.*, 1988b; Hawley-Nelson *et al.*, 1989). As mentioned before, p53 and pRB are major substrates for E6 and E7 in context of their immortalizing activity, however the interaction and perturbation of additional cellular factors are required for the full transforming capacity of HPV oncoproteins. For example, in tissue culture experiments E6 with mutated PBM was less efficient in conferring EMT-like characteristics and, moreover, was defective in cellular transformation and tumorigenicity (Kiyono *et al.*, 1997; Watson *et al.*, 2003). Furthermore, the HPV 16 E6 PBM was also shown to be required for induction of anchorage-independent growth of human tonsillar keratinocytes in cooperation with an activated Ras oncoprotein (Spanos *et al.*, 2008a; Spanos *et al.*, 2008b).

An important contribution towards the understanding of the relative contribution of E6 and E7 in malignant transformation has been provided by transgenic animal models mimicking the natural progression of HPV-induced tumorigenesis. In this model, transgenic mice express the K14HPV16 E6/E7 transgene, in which E6 and E7 are placed under the control of the human keratin 14 (K14) promoter, whose activity is restricted to cells occupying the basal layer of the stratified epithelium. This restricts the expression of E6 and E7 to the cell type in which the natural infection takes place. Mice expressing the entire HPV early region under the control of the K14 promoter displayed hyperplasia, dysplasia and papillomatosis in different epidermal and mucosal sites (Arbeit *et al.*, 1994). In addition, the individual expression of E6 or E7 promoted epithelial dysplasia and skin tumors. It is interesting to note that tumors developing from E7-expressing mice were significantly different from those induced by E6 expression. The expression of E7 induced benign and well differentiated tumors, conversely those expressing E6 had a more malignant phenotype (Simonson *et al.*, 2005; Song *et al.*, 2000).

In contrast with animal models for skin carcinogenesis, transgenic mouse models for cervical cancer suggested that E7 can drive initial proliferation and induction of primary cervical tumors with weak invasive capacity, whilst E6, although it fails to drive the initial steps of tumorigenesis,

significantly increases the dimension as well as the invasiveness of primary tumors induced by E7 (Riley *et al.*, 2003). These results were also supported by studies in models for head and neck squamous cell carcinoma (HNSCC) and in co-carcinogen assays, in which transgenic mice were treated with chemical carcinogens known to drive different stages of carcinogenesis. In these studies E7 was found to retain the strongest transforming potential and to drive tumorigenesis, whereas E6 had weak transforming capacity and was dispensable for initial tumor formation, although E6 contributed significantly in the later stages of tumor progression, enhancing the malignant conversion of the primary tumor (Strati and Lambert, 2007; Song *et al.*, 2000).

Mutational analysis of E6 in the transgenic mouse models has shown that lack of p53 failed to cooperate with carcinogens to produce epidermal hyperproliferation, suggesting the existence of p53-independent tumorigenic pathways used by E6 (Song *et al.*, 1999). In addition, mice expressing a mutant form of E6, E6I128T, unable to interact with E6AP, showed a reduced ability to overcome DNA synthesis block upon exposure to ionizing radiation, and to have a decreased propensity to develop spontaneous skin tumors compared with wild-type K14 E6 mice (Nguyen *et al.*, 2002b). This also supports the notion that the association of E6 with E6AP is not exclusively required for the degradation of p53, but is involved in the inactivation of additional targets. Good candidates for p53-independent substrates important for E6 tumorigenic activity are the PDZ domain-containing proteins. Consistent with this, in mice expressing a PBM-defective mutant of E6 (E6Δ146-151), which retains the ability to interact with p53, but displays a reduced ability to promote hyperplasia in squamous epithelia, E6Δ146-151 still retained the capacity to promote malignant progression (Nguyen *et al.*, 2003a; Simonson *et al.*, 2005). This observation was also consistent with similar experiments in mouse lens epithelia, where the PBM of E6 was required to induce epithelial hyperplasia and defects in cell adhesion and differentiation (Nguyen *et al.*, 2003a). In HPV-16 cervical cancer transgenic mouse models, expression of a PBM mutant of E6 in combination with wild type E7 led to the formation of smaller tumors characterised by a reduced invasive potential (Shai *et al.*, 2007). Thus the interaction of E6 with PDZ-containing substrates has marked effects on the metastatic progression of HPV-related cancers in mice.

Results

Part I:

Analysis of PDZ-containing proteolytic substrates of E6 in HPV-positive cells

MAGI-1, hDlg and hScrib are degraded by E6 in HeLa and CaSKi Cells

As mentioned before, several studies have identified a number of PDZ domain-containing proteins as potential targets of the high-risk HPV E6 proteins. Many of them were analysed in ectopic overexpression systems and each study made use of different cell types, thus making it difficult to directly compare the susceptibility of these targets to E6-induced degradation in cells. In an attempt to compare directly the degree to which the various PDZ domain targets of E6 are susceptible to increased rates of degradation, we used siRNA to block E6/E7 expression in CaSKi (HPV-16 positive) and HeLa (HPV-18 positive) cells. HeLa and CaSKi are two cervical cancer-derived cell lines, hence they can reflect more closely the molecular events brought about by the expression of E6 and E7 in the context of cervical carcinogenesis. Extracts from E6/E7-silenced cells were analysed by western blot and compared with those from control cells transfected with siRNA to Luciferase, or from cells transfected with siRNA to E6AP. In the first set of assays we analysed changes in the levels of expression of hScrib and hDlg at 48h and 72h post-transfection. The results in Figure 10 show that there is a strong increase in the levels of p53 at both time points following siRNA transfection against E6/E7 and E6AP in both CaSKi and HeLa cells. This is in agreement with previous studies (Scheffner *et al.*, 1993; Huibregtse *et al.*, 1993) and confirms efficient ablation of E6 expression. In the case of hDlg, there is a modest increase in expression at the 48h time point in HeLa cells, and this is even more apparent at the 72h time point, thus confirming hDlg as a substrate for HPV-18 E6-induced degradation in HeLa cells (Mantovani *et al.*, 2001a; Massimi *et al.*, 2004; Handa *et al.*, 2007). Interestingly there is also a slight increase in the overall level of hDlg expression in CaSKi cells. It is also noteworthy that hDlg migrates somewhat differently in the two cell lines, most likely reflecting differences in the degree of phosphorylation. This is particularly relevant, since phosphorylation events can influence the pattern of hDlg

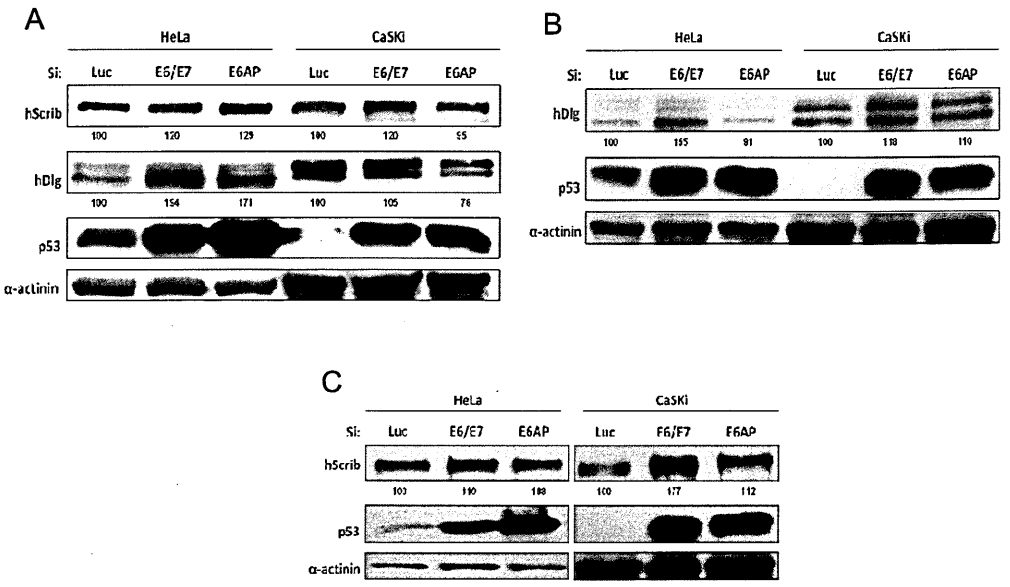

Figure 10. hDlg and hScribble are degraded in HeLa and CaSKi cells. A. HPV-positive HeLa and CaSKi cells were transfected with siRNA Luciferase, siRNA E6/E7 or siRNA E6AP and grown for 48h prior to harvesting. The expression patterns of hDlg, hScribble, p53 and also α-actinin to monitor the protein loading, were assessed by western blot analysis. B and C. The assay was repeated as in A, but HeLa and CaSKi cells were harvested at 72h post- transfection before western blot analysis. Numbers represent the percentage of band intensity for hScribble and hDlg in comparison with the siRNA Luciferase control (100%).

localization and its susceptibility to E6-mediated degradation (Massimi *et al.*, 2006; Narayan *et al.*, 2009b). In the case of hScrib, there is a significant increase in its levels of expression in the E6/E7 siRNA treated CaSKi cells at both the 48h and 72h time points, whereas changes in hScrib levels in HeLa cells are minimal at both time points. E6AP ablation has only modest effects on both hDlg and hScrib, which are somewhat dependent upon the time at which the assays were performed, however there are slight increases in hDlg expression in HeLa cells (Figure 10a) and in hScrib expression in CaSKi cells (Figure 10c). Taken together, these results confirm previous studies suggesting that hDlg and hScrib are preferential proteolytic substrates of HPV-18 and HPV-16 E6 respectively (Thomas *et al.*, 2005b).

Previous studies showed that the Dlg-related protein MAGI-1 is strongly bound by high-risk E6 oncoproteins, and is also susceptible to E6-induced degradation (Glaunsinger *et al.*, 2000; Thomas *et al.*, 2001). However these studies were also performed in an *in vitro* setting or under conditions of ectopic expression, and the effects of E6 ablation on MAGI-1 expression in HPV-positive cells were not determined. Therefore, we analysed the MAGI-1 pattern of expression in a similar series of E6/E7 and E6AP ablation experiments in HeLa and CaSKi cells. The results in Figure 11 demonstrate a strong increase in the levels of MAGI-1 expression in E6/E7-silenced HeLa cells at 24 and 48h post-transfection, and this is also observed equally well following ablation of E6AP expression. In the case of CaSKi cells, there is a modest increase in MAGI-1 levels, although not as strong as that observed in HeLa cells. Since the rescue of MAGI-1 in CaSKi was weak, we wanted to confirm that the band detected did correspond to the MAGI-1 protein. To do this, we repeated the analysis including an siRNA against MAGI-1. The results obtained are shown in Figure 11c and, as can be seen, the protein that is rescued following siRNA against E6/E7 disappears when the MAGI-1 siRNA is also included. We then extended the analysis to another HPV-16 positive cell line, SiHa, and obtained similar results (Figure 11d). Taken together, these results would suggest that whilst MAGI-1 is a good substrate of HPV-18 E6 it is also subject to HPV-16 E6-induced degradation.

Analysis of FAP1, TIP2, PTPN3 and PSD95 expression levels following E6/E7 knockdown

We next wanted to determine whether other reported PDZ substrates of E6, such as FAP-1, TIP-2, PTPN-3 and PSD95, were also similarly targeted by E6 in cervical tumour-derived cell lines. The HeLa and CaSKi cells were transfected with siRNA against E6/E7 and changes in the PDZ protein levels ascertained by western blot analysis. The results in Figure 12 show no significant changes in the levels of expression of either PTPN3 (Figure 12a) or TIP2 (Figure 12c), following siRNA ablation of either E6/E7 or E6AP. In contrast there was a significant increase in the levels of expression of PSD95 in HeLa cells and a slight increase in CaSKi cells (Figure 12b) following E6/E7 knockdown. Removal of E6AP had no effect on PSD95 levels of expression in HeLa cells, whilst in CaSKi cells, a greater increase was obtained. These results confirm that PSD95 is a potential target for HPV-18 and HPV-16 E6-induced degradation (Handa *et al.*, 2007). In the case of FAP1, an apparently contradictory result was obtained. Although its levels of expression in HeLa cells are very low, siRNA to E6/E7 apparently reduces FAP1 levels still further (Figure 12d). This effect is more marked in CaSKi cells, where loss of E6/E7 expression results in a dramatic decrease in the levels of FAP1 expression. In addition, removal of E6AP also results in lower levels of FAP1 expression in HeLa, with a slight decrease also evident in CaSKi cells.

Since a number of these proteins were present at quite low levels, we also verified that the correct proteins were detected by the antibodies. To do this we performed a series of western blot analyses on cell extracts following transfection with siRNAs to each of the PDZ-domain containing proteins. The results in Figure 12e (PTPN3, TIP2 and FAP1) and Figure 12b (PSD 95) show that the protein recognized by the relevant antibody also disappears following transfection with the relevant siRNA.

Taken together, these results demonstrate that PTPN3 and TIP2 are not major targets of E6-induced degradation in monolayer cultures of cells derived from cervical tumours. In contrast, PSD95 appears to be a good substrate for HPV-18 E6, in agreement with previous studies (Handa *et al.*, 2007). Finally, it would appear that, at least in cervical tumour-derived cells in monolayer culture,

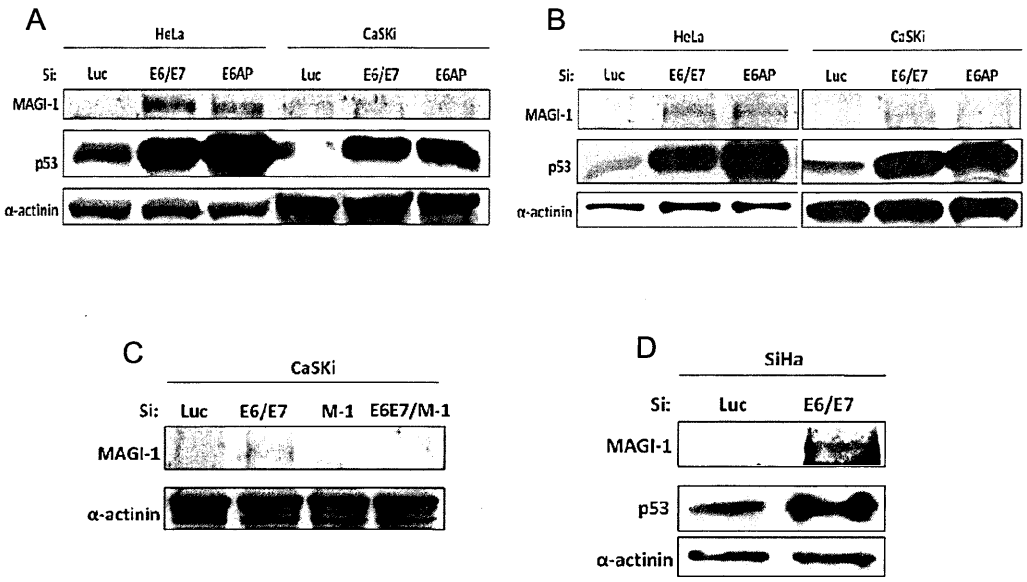

Figure 11. MAGI-1 is efficiently rescued in HPV-16- and HPV-18-positive cells upon E6/E7 ablation. A. HeLa and CaSKi cells were transfected with siRNA Luciferase, siRNA E6/E7 or siRNA E6AP and grown for 48h before harvesting. The expression patterns of MAGI-1, p53 and also α-actinin to monitor the protein loading, were assessed by western blot analysis. B. The assay was repeated as in A, but cells were grown for 72h before harvesting and western blot analysis. C. To confirm the identity of the band corresponding to MAGI-1, CaSKi cells were transfected with siRNA Luciferase, siRNA E6/E7, siRNA MAGI-1 (M-1) or a combination of siRNA E6/E7 and siRNA MAGI-1. 72h after transfection cells were harvested and western blot analysis was performed as in A and B. D. The assay was repeated in SiHa cells and processed as in panel B.

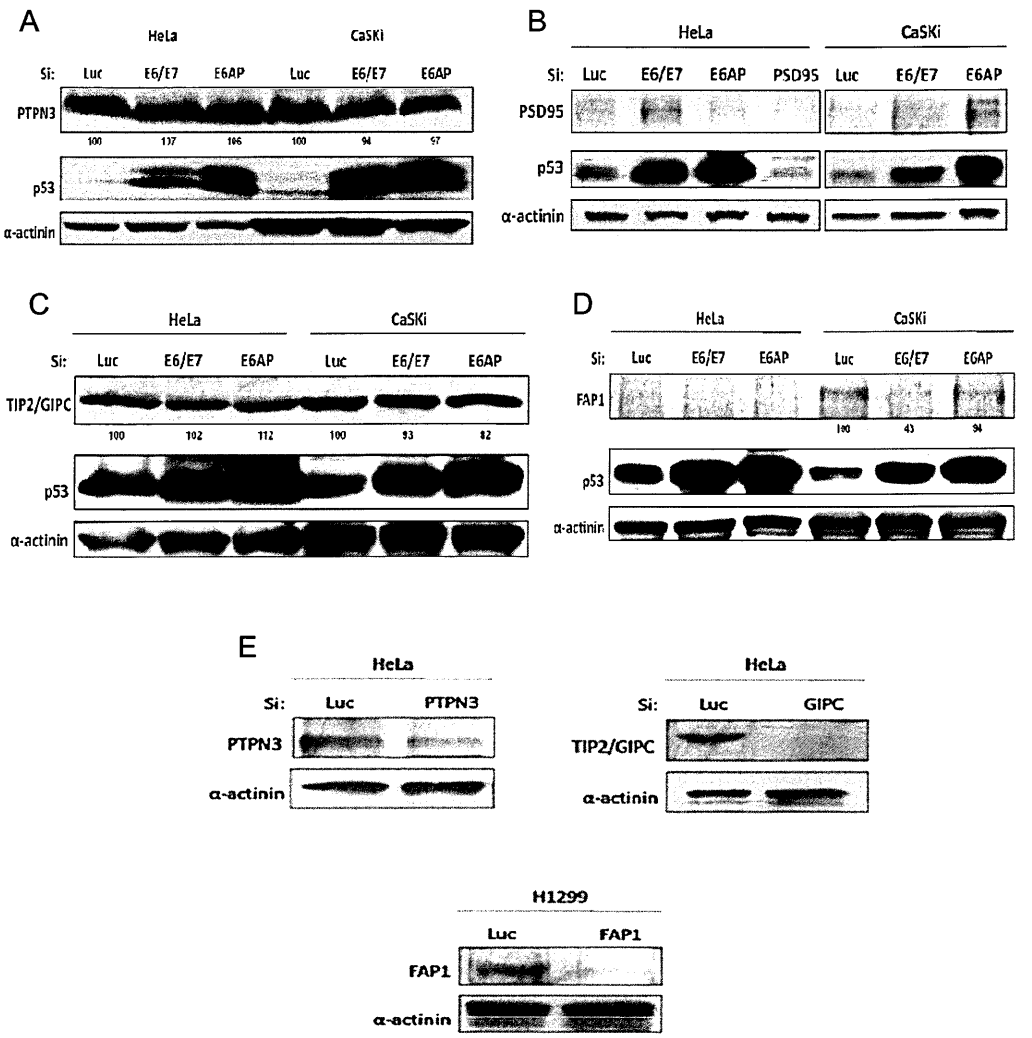

Figure 12. Analysis of PTPN3, PSD95, TIP2/GIPC and PTPN13/FAP1 susceptibility to E6 degradation *in vivo*. See the next page for the full legend.

Figure 12 (Cont.). HeLa and CaSKi cells were transfected with siRNA Luciferase, siRNA E6/E7 or siRNA E6AP and grown for 72h before harvesting. The expression levels of PTPN3 (A), PSD95 (B), TIP2/GIPC (C), PTPN13/FAP1 (D), p53 and α-actinin, were assessed by western blot. E. To confirm the correct identity of PTPN3, TIP2 and PTPN13, cells were transfected with siRNA Luciferase, siRNA PTPN3, siRNA TIP2/GIPC, or siRNA PTPN13/FAP1 and their expression patterns were assessed by western blot analysis. In panels A-D, numbers represent the percentage of band intensity for PTPN3, PSD95, TIP2 and FAP1 in comparison with the siRNA Luciferase control (100%).

E6/E7 might actually increase the levels of FAP1 expression. Whether this is through E6's PDZ interactions with FAP1, or through an, as yet, unknown function of E7 remains to be determined.

HPV E6 preferentially degrades nuclear and membrane bound pools of MAGI-1

Previous studies have shown that certain cellular pools of hDlg are more susceptible than others to E6-induced degradation (Massimi *et al.*, 2004; Massimi *et al.*, 2006; Narayan *et al.*, 2009). Since MAGI-1 is a major common target for both HPV-16 and HPV-18 E6, we were interested in determining whether there are also similar cellular pools of MAGI-1 that are preferentially targeted by E6. To investigate this HeLa cells were transfected with siRNA against E6 and E7, and after 72 hours cells were harvested and subjected to differential sub-cellular fractionation. For comparison we also included H1299 cells in the analysis to determine where MAGI-1 would normally be expressed in epithelial cells in the absence of HPV sequences. The cells were fractionated into cytosolic, membrane, nuclear and cytoskeletal components, and the levels of MAGI-1 expression in each fraction were ascertained by western blot analysis. The results obtained again demonstrate that MAGI-1 is a strong substrate for HPV-18 E6 induced degradation in HeLa cells (Figure 13a). Interestingly, the bulk of MAGI-1 protein that is rescued upon ablation of E6/E7 expression resides mainly in the membrane and nuclear fractions of the cell, with the largest recovered pool actually being present within the nucleus. In contrast, a similar fractionation of H1299 cells (Figure 13b) shows that the main concentration of MAGI-1 is found at membrane sites, with slightly smaller pools in the nuclear and cytosolic fractions. These studies demonstrate that the rescue of MAGI-1 from E6-induced degradation results in a preferential restoration of MAGI-1 expression at membrane sites and also within the nucleus, suggesting that E6 targets MAGI-1 for degradation at membrane and nuclear sites.

HPV E6-induced degradation of MAGI-1 disrupts cellular TJs

Although there is no information on the potential function of MAGI-1 in the nucleus, previous studies have implicated the membrane-bound form of MAGI-1 in the establishment of cellular TJs (Murata *et al.*, 2005). It has also been shown that TJs are disrupted in HPV-positive cells, and a

possible role for hScrib was suggested in this phenotype (Nakagawa and Huibregtse, 2000). However, we reasoned that MAGI-1 was also a likely candidate to explain the disruption of TJs by HPV E6, since its expression is required to promote the junctional targeting of TJ-associated proteins, including ZO-1 and occludin (Hirabayashi *et al.*, 2003). Therefore we proceeded to investigate the TJ status in cells that had been ablated for E6/E7 expression. At the same time we performed siRNA ablation of MAGI-1 and of hScrib on a subset of cells treated with siRNA to E6/E7, to determine whether any changes in TJs were MAGI-1 or hScrib dependent. 72h and 96h post-transfection of the siRNAs, HeLa cells were fixed and analysed by immunofluorescence for MAGI-1 and a TJ marker, ZO-1 (Stevenson *et al.*, 1986, Denker and Nigam, 1998). We focused primarily on cells that were in contact so that junctions would have the opportunity to become established, and the results for MAGI-1 are shown in Figure 14. As can be seen, there is no MAGI-1 protein detectable at sites of cell-cell contact in the siRNA Luciferase control cells and ZO-1 displays a diffused pattern of expression and is also absent at these sites. In contrast, siRNA to E6/E7 results in a very marked accumulation of MAGI-1 expression at cell-cell junctions. Interestingly, this occurs in a beaded structure at the 72h time point and there is also a perfect co-localisation with ZO-1 in these structures, suggesting the re-initiation of correct TJ formation. By the 96h time point this is even more marked and a broader junctional staining could be observed between adjacent cells. These results demonstrate that TJs can be re-established in HeLa cells when E6/E7 expression is ablated. Interestingly, a similar pattern of staining is also obtained upon ablation of E6AP expression, consistent with the results from the western blot analyses. We also analysed cells that had been co-transfected with a MAGI-1 siRNA, and, as can be seen in Figure 14, there is a failure to restore TJs in cells treated with the MAGI-1 siRNA, as determined by the pattern of ZO-1 expression, even by the 96hr time point. In contrast, ablation of hScrib (Figure 15a) has no effect upon the re-establishment of TJs upon ablation of E6/E7 expression. In addition, removal of E6/E7 expression in HeLa cells results in a marked increase in hScrib expression at the cell membrane, despite the apparent lack of significant levels of degradation seen in Figure 10. Furthermore, in control siLuciferase CaSKi cells residual hScrib is localized predominantly at cell contact sites, and a potent rescue of the cytoplasmic pool of the protein can be observed upon

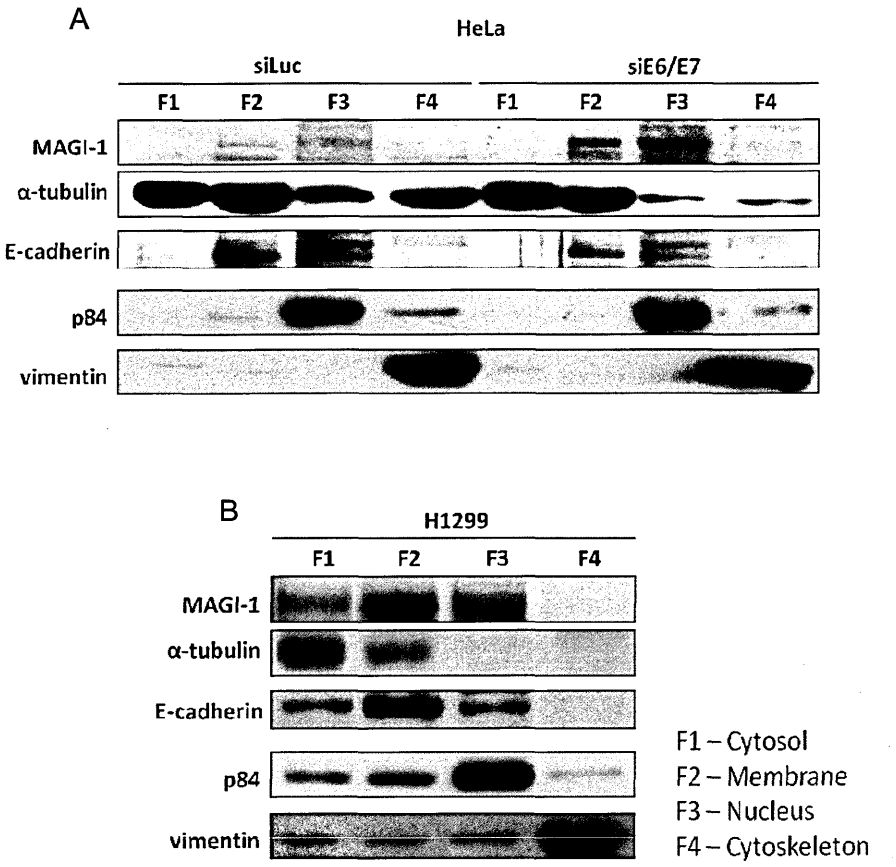

Figure 13. MAGI-1 is rescued at membrane and nuclear sites upon silencing of E6 in HPV-positive cells. A. HeLa cells were transfected with siRNA Luciferase or siRNA E6/E7, and after 72h cells were fractionated into 4 subcellular compartments: cytosol (F1), membrane (F2), nucleus (F3), cytoskeleton (F4). The expression patterns of MAGI-1 or those of the four subcellular fraction markers E-cadherin, nuclear matrix protein p84, α-tubulin and vimentin were assessed by western blot analysis. B. The subcellular fractionation was repeated on HPV-negative H1299 cells and the levels of MAGI-1 and of the subcellular fraction markers were detected as in A.

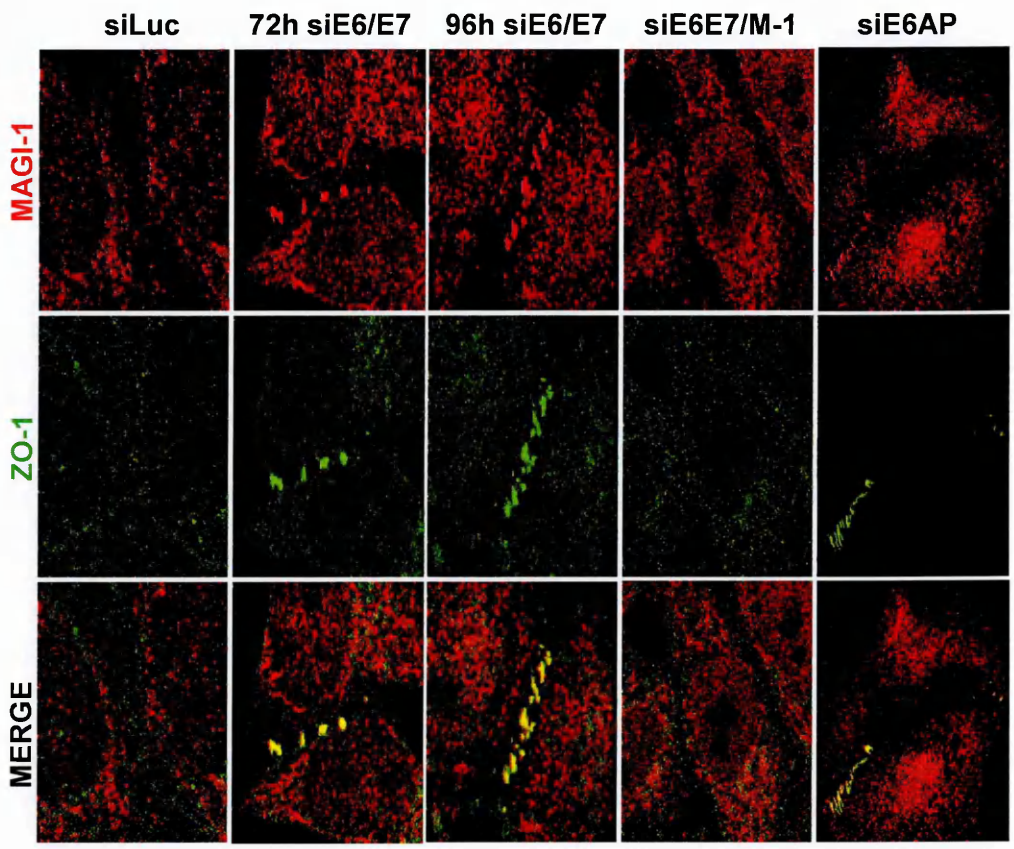

Figure 14. Rescue of MAGI-1 from E6-induced degradation restores TJs. HeLa cells were seeded on glass coverslips and transfected either with siRNA Luciferase, siRNA 18E6/E7, a combination of siRNA 18E6/E7 plus siRNA MAGI-1, or with siRNA E6AP. Cells were grown for 72h or 96h before fixing and incubated with anti-MAGI-1 and anti-ZO-1 antibodies and counterstained with rhodamine-conjugated (MAGI-1) and fluorescein-conjugated (ZO-1) secondary antibodies. Confocal images were taken at 480 and 510 nm wavelengths.

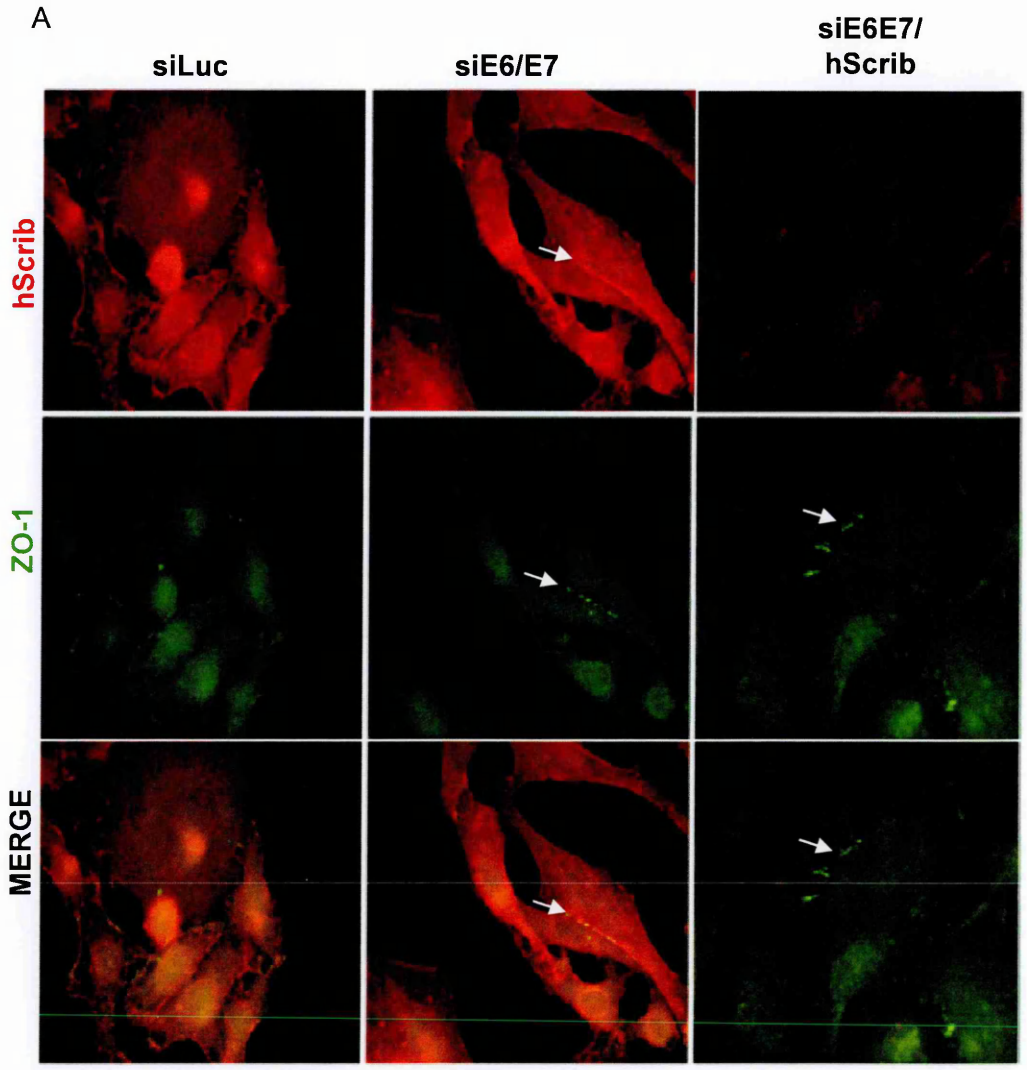

Figure 15. Loss of hScrib does not affect TJ formation. See the next page for the full legend.

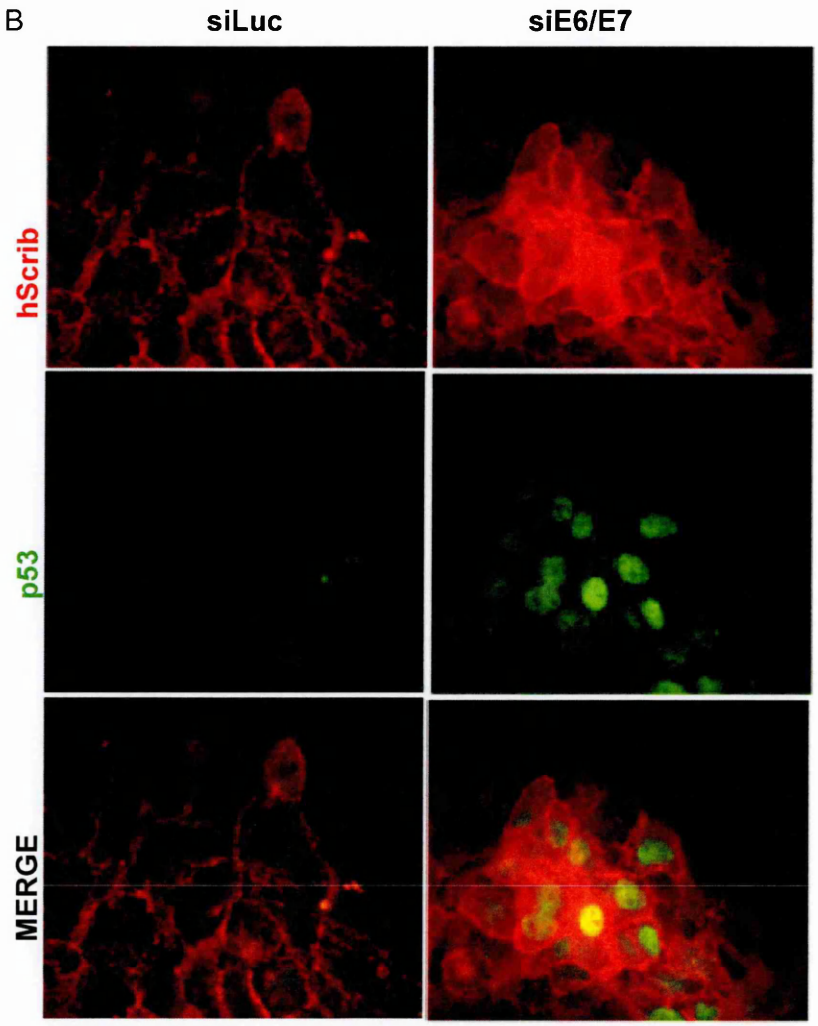

Figure 15 (Cont.). Loss of hScrib does not affect TJ formation. HeLa cells (panel A) and CaSKi cells (panel B) were seeded on glass coverslips and transfected with siRNA Luciferase, siRNA 18E6/E7, siRNA 18E6/E7 in combination with siRNA hScrib or siRNA 16E6/E7 as indicated. Cells were grown for 96h before fixing and staining for hScrib (red) and ZO-1 (green) in HeLa cells or for hScribble (red) and p53 (green) in CaSKi cells. White arrows indicate sites of hScrib and ZO-1 junctional accumulation.

silencing of E6 and E7 (Figure 15b), suggesting that hScrib can be differentially regulated by HPV-16 or -18 E6 oncoproteins. However, taken together, these results suggest that loss of TJs in a HPV-positive, tumour-derived cell line is, at least in part, due to the ability of E6 to induce the degradation of MAGI-1.

Generation of an E6-resistant MAGI-1 mutant

The K499E mutation reduces MAGI-1 affinity for E6

The fact that MAGI-1 is a sensitive proteolytic substrate of E6 prompted us to perform a more detailed analysis of its possible function in the context of HPV pathology. To do this, we reasoned that using a MAGI-1 mutant that was resistant to E6 targeting might by one way to address this. We made use of previous studies suggesting that E6 interacts with MAGI-1 specifically through its PDZ domain 1 (Thomas *et al.*, 2001), and more recent structural data that identified the lysine 499 (K499) as one of the most critical residues within the PDZ1 domain mediating its interaction with E6 (Fournane *et al.*, 2011). Using surface plasmon resonance to measure the binding affinity between peptides encompassing the HPV-16 and -18 E6 PBMs and a purified form of MAGI-1 PDZ1, these studies suggested that the K499E mutation was indeed able to produce a dramatic decrease in the interaction between E6 and MAGI-1 (Fournane *et al.*, 2011). However, these results were not verified in the context of full-length E6 and MAGI-1 proteins and, importantly, the impact of the K499E mutation on the ability of E6 to degrade MAGI-1 was not assessed. In an attempt to address these points, we decided to introduce the K499E mutation into the full-length MAGI-1 cDNA. For this purpose, a FLAG-tagged wild-type MAGI-1 (M1wt) expression construct was used as a template to generate the K499E MAGI-1 mutant (M1K499E) by site-directed mutagenesis. Figure 16a shows a comparison between the protein sequence of wild-type and K499E MAGI-1 PDZ1 domain, and the structural elements of the PDZ domain that mediate the interaction of MAGI-1 to its PBM-containing partners are highlighted. Once the FLAG-tagged M1K499E was generated, we first wanted to verify that the wild-type and mutant MAGI-1 proteins were expressed at comparable levels. To assess this, 293 cells were transiently transfected with the two constructs. 24h after transfection cells were harvested and MAGI-1 expression levels were analysed by SDS-

PAGE and western blot using anti-FLAG antibody. As can be seen in Figure 16b, wt and mutant MAGI-1 proteins are expressed at comparable levels in 293 cells, suggesting that the K499E mutation does not alter the steady state levels of MAGI-1. In order to test whether the K499E mutation could indeed reduce the affinity of MAGI-1 for HPV-16 and -18E6 oncoproteins, we first performed GST pull-down assays. To do this, HPV-16 and 18 E6 were expressed as GST-fusion proteins and purified using glutathione-coated agarose beads. The beads were incubated for 1 hour at 4°C with cell extracts from 293 cells that had been transiently transfected with either the M1wt or M1K499E expression plasmids. After extensive washing, the amount of MAGI-1 bound to GST-E6 was assessed by SDS-PAGE and western blotting using anti-FLAG antibody. As can be seen in Figure 16c, GST-18 E6 bound strongly to M1wt and bound about 70% of the protein compared to the input, whereas the K499E mutation exhibited a dramatically reduced ability to interact with E6. Similar results were also obtained with GST-16 E6 (Figure 16d), although wild-type MAGI-1 was bound less efficiently compared with 18 E6, which is in agreement with previous studies (Thomas *et al.*, 2001). Thus these results confirm that the residue K499 within the MAGI-1 PDZ1 domain is important for the recognition of MAGI-1 by both the HPV-16 and -18 E6 oncoproteins.

MAGI-1 K499E mutant is resistant to E6-mediated degradation

To determine whether the reduced ability of E6 to interact with the MAGI-1 K499E mutant also resulted in decreased rates of degradation, we compared the relative susceptibilities of wild-type and mutant MAGI-1 to E6-mediated degradation *in vitro*. The two MAGI-1 proteins were *in vitro* translated in the presence of ^{35}S-labelled methionine, and then incubated at 30°C for different periods of time with *in vitro* translated and radiolabelled HPV-16 and -18 E6, and the pattern of MAGI-1 expression was assessed by SDS-PAGE and autoradiography. The results in Figure 17a show that the levels of wild-type and mutant MAGI-1 are stable over a period of 120 minutes when incubated in absence of E6. In contrast, wild-type MAGI-1 was degraded upon addition of HPV-16 or -18 E6, with HPV-18 E6 being the most efficient (Thomas *et al.*, 2001). Consistent with the reduced level of interaction, M1K499E was also significantly more resistant to E6-mediated

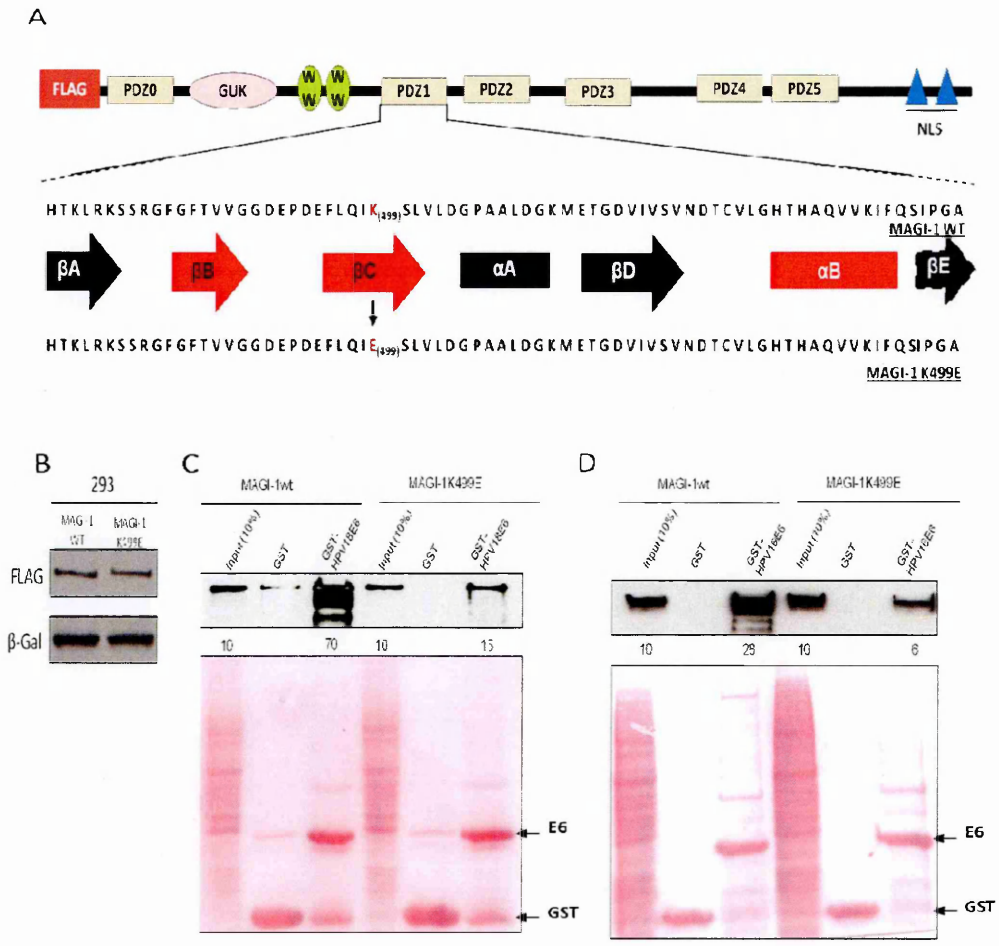

Figure 16. The K499E mutation affects the E6-binding capacity of MAGI-1. See the next page for the full legend.

Figure 16 (Cont.). The K499E mutation affects the E6-binding capacity of MAGI-1. A. diagram showing the domain composition of MAGI-1 and the location of the K499E mutation. Elements of secondary structure that compose the PDZ1 are also shown (βA-E (β-strands A to E); αA-B (α-helix A to B)), and those involved in the interaction with E6 and other target proteins are highlighted in red (adapted from Fournane et al., 2011). B. HEK 293 cells were transfected with 1μg of FLAG-tagged wild-type or mutant MAGI-1 and grown for 24h hours prior to harvesting. The levels of MAGI-1 expression were assessed by western blot. β-galactosidase was included to monitor the transfection efficiency. C and D. Extracts from HEK293 cells transfected with 3 μg of wild-type or mutant MAGI-1, were subjected to GST-pulldown reactions with the indicated GST fusion proteins, and bound MAGI-1 was detected by western blot using anti-FLAG antibody. Numbers represent the percentage of wild type and mutant MAGI-1 proteins bound to the indicated GST-fusion protein relative to the input control. The lower panel shows the Ponceau staining of the membrane, confirming the equal loading of the GST proteins.

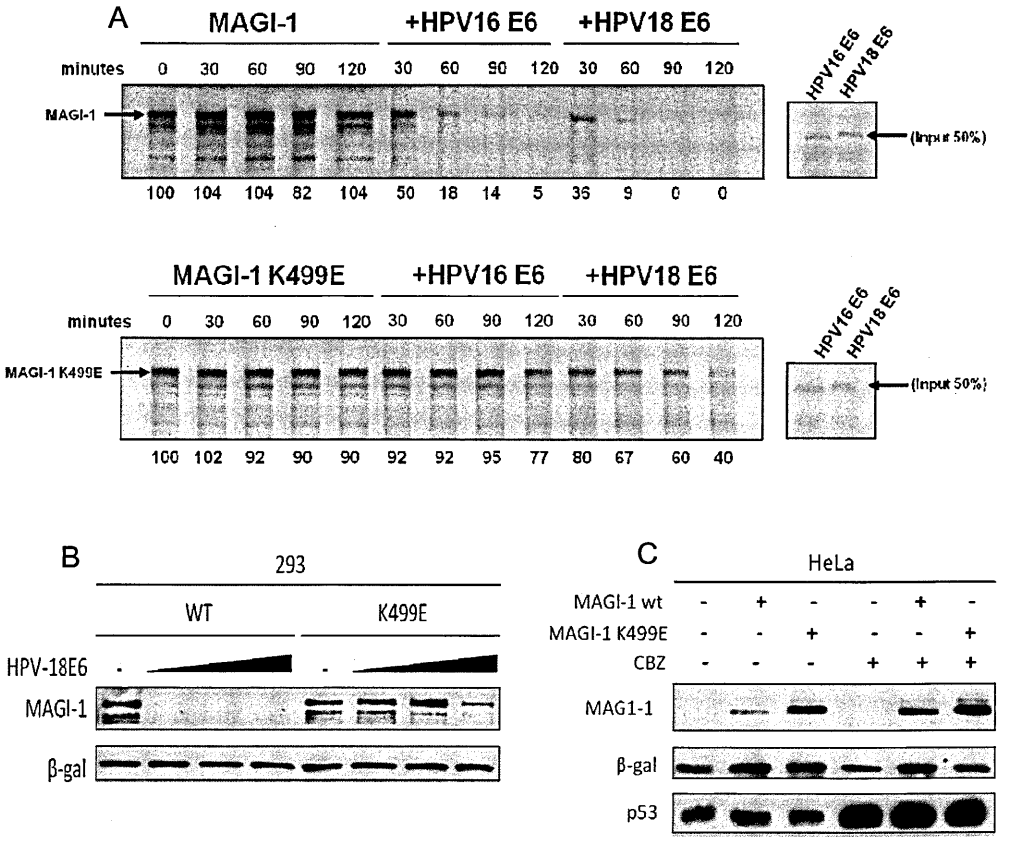

Figure 17. The K499E mutation renders MAGI-1 resistant to E6-mediated degradation. See the next page for the full legend.

Figure 17 (Cont.). A. Wild-type and the K499E mutant, together with HPV-16 and HPV-18 E6 oncoproteins, were in vitro translated in the presence of ^{35}S-labelled methionine or cysteine. They were then incubated together at 30°C as indicated. Residual MAGI-1 protein was detected by SDS-PAGE and autoradiography. Numbers are the band intensities expressing the percentage of residual MAGI-1 protein relative to the control (100%). B. HEK 293 cells were co-transfected with 1μg of FLAG-tagged wild-type or K499E mutant and either 2, 5 and 10μg of HPV-18 E6 expression plasmid. After 24 hours cells were harvested and the expression levels of MAGI-1 and β-galactosidase were detected by western blot analysis. C. HeLa cells were transfected with 3μg of FLAG-tagged wild-type or K499E mutant MAGI-1. After 24 hours the cells were treated with MG-132 for an additional 3 hours prior to harvesting. The expression levels of MAGI-1, p53 and β-galactosidase were assessed by western blot analysis.

degradation; although low levels of degradation were observed at the later time points, which is consistent with the residual binding of E6 to the K499E mutant.

To investigate whether similar results could also be obtained *in vivo*, we compared the steady state levels of FLAG-tagged wild-type and mutant MAGI-1 in 293 cells when expressed alone or in combination with increasing amounts of HPV-18 E6 (Figure 17b). In good agreement with the *in vitro* assay, M1wt was highly susceptible to E6-induced degradation, whereas, the K499E mutant was significantly more resistant. We then proceeded to investigate whether the K499E mutant was resistant to E6 targeting in the more physiologically relevant setting of cervical cancer-derived cells that express endogenous levels of E6 oncoprotein. To do this we expressed the FLAG-tagged MAGI-1 constructs in HeLa cells. 24 hours after transfection, cells were left untreated or treated with the proteasome inhibitor MG-132 for an additional 3 hours before harvesting, in order to determine whether differences in MAGI-1 level of expression were due to proteasomal degradation. Total cell extracts were separated by SDS-PAGE and the pattern of MAGI-1 and, for comparison, of p53 expression was analysed by western blot. As can be seen in Figure 17c, MG-132 treatment produced a strong recovery in the levels of p53 expression, confirming the efficient inhibition of the proteasome. In agreement with our degradation assays in 293 cells, the expression levels of the K499E mutant in HeLa were significantly higher than the wild-type protein, again reflecting their differential susceptibility to HPV-18 E6-mediated degradation. Proteasome protection produced an increase in the levels of expression of both M1wt and M1K499E, confirming that M1K499E is nonetheless susceptible to 18 E6-mediated degradation, although to a much lesser extent than the wild-type MAGI-1.

The K499E mutation perturbs the functionality of MAGI-1 PDZ1 but does not affect its sub-cellular localization

The crystal structures of MAGI-1 PDZ1 and Dlg PDZ2 and 3 in complex with a peptide encompassing the HPV-18 E6 PBM have been solved (Zhang *et al.*, 2007). These data suggested that, unlike most PDZ-PBM interactions, the association of 18 E6 with PDZ domains is stabilized

by non-canonical residues that extend upstream of the PBM, and make contact with β-strands B and C and the α-helix B of PDZ domains (Zhang *et al.*, 2007; Thomas *et al.*, 2008a). With regard to the MAGI-1 PDZ1 domain, the K499 residue maps in its β-strand C (Figure 16a), one of the structural components in close proximity to the substrate binding groove (Doyle *et al.*, 1996; Fournane *et al.*, 2011), indicating that the alteration of this domain could potentially perturb the functionality of the PDZ domain rather than selectively block the interaction with E6. To date, several PBM-containing cellular proteins have been identified as potential binding partners for MAGI-1 PDZ1, including the RhoA modulators NET1 and p116Rip (Dobrosotskaya, 2001), JAM4 (Hirabayashi *et al.*, 2003) and the zyxin family protein LPP (Fournane *et al.*, 2011). Of these, however, only the interactions between MAGI-1 PDZ1 and the Rho GEF NET1 and JAM4 have been confirmed by biochemical analysis (Dobrosotskaya., 2001; Hirabayashi *et al.*, 2003), although JAM4 has been shown to preferentially associate with MAGI-1 through its PDZ4 domain (Hirabayashi *et al.*, 2003). In an attempt to define whether the K499E MAGI-1 mutant retained the overall functionality of its PDZ1 domain, we decided to monitor the interaction of M1wt and M1K499E with NET1. To do this, 293 cells were transiently transfected with the FLAG-tagged M1wt and M1K499E constructs, either alone or in combination with MYC-tagged NET1. After 24 hours, the cells were harvested and cell extracts were immunoprecipitated using anti-MYC antibody, and the co-immunoprecipitated M1wt and M1K499E were detected by western blot using anti-FLAG antibody (Figure 18a). The results of these assays demonstrate that wild-type MAGI-1 specifically co-immunoprecipitates with NET1, confirming previous results (Dobrosotsakaya, 2001). In contrast, the interaction between NET1 and the K499E mutant was significantly reduced, suggesting that this mutation does introduce structural changes into the substrate binding groove of the PDZ1 domain so that the ability of MAGI-1 PDZ1 to interact with the PBM of NET1 is perturbed. It is interesting to note, however, that the PBMs of 16 and 18 E6 oncoproteins, as well as that of NET1, possess a glutamic acid at position -3 which contributes to the interaction with K499 within the MAGI-1 PDZ1 domain (Figure 18b). Therefore, we reasoned that the interaction of the MAGI-1 PDZ1 domain with PBMs that do not have a glutamic acid at the -3 position might be less susceptible to the K499E mutation. In order to test this hypothesis, we repeated the GST pull-down

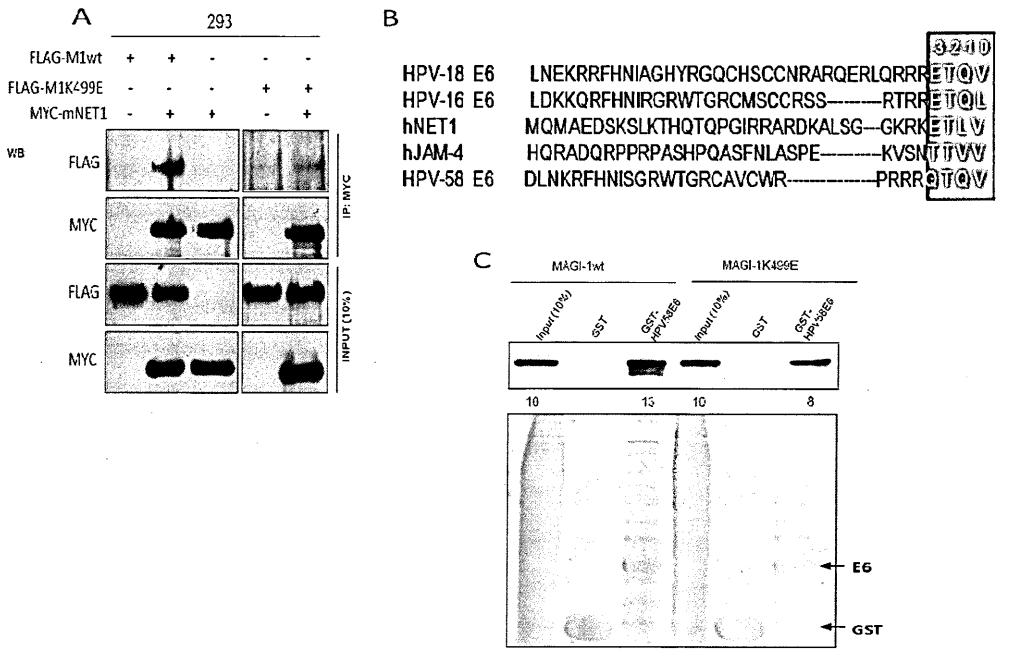

Figure 18. The K499E mutation affects the interaction of MAGI-1 with NET1 but not with HPV-58 E6. A. HEK293 cells were transfected with 3μg of the FLAG-tagged MAGI-1 plasmids together with 5μg of MYC-tagged mNET1, as indicated. After 24 h cells were extracted and immunoprecipitated using anti-MYC antibody. NET1-bound MAGI-1 moieties were then detected by western blot using anti-FLAG antibody. B. Sequence alignment of the PBMs of JAM-4, NET1, HPV-16 E6, HPV-18 E6 and HPV-58 E6. C. HEK 293 cells were transfected with 3μg of the FLAG-tagged MAGI-1 constructs; after 24 h the cells were harvested and extracts were subjected to GST-pulldown reactions using the GST-HPV-58 E6 fusion protein, and bound MAGI-1 was detected by western blot using anti-FLAG antibody. Numbers represent the percentage of wild type and mutant MAGI-1 proteins bound to the indicated GST-fusion protein relative to the input control. The lower panel shows the Ponceau staining of the membrane, confirming the equal loading of the GST proteins.

assays of wild type and mutant MAGI-1 using purified GST-tagged HPV-58 E6, whose PBM is identical to HPV-18 E6 except that it has a glutamine at the -3 position (Figure 18b). As shown in Figure 18c, the amount of wild type and K499E mutant MAGI-1 pulled down by HPV-58 E6 was similar. This suggests that the K499E mutation only marginally affects the ability of HPV-58 E6 to bind to MAGI-1. Therefore, this confirms that the presence of a glutamic acid at the -3 position of the PMB of MAGI-1-binding partners makes an important contribution to the interaction, and, importantly, also suggests that the K499E mutation might affect to a lesser extent the interaction with other MAGI-1 binding partners that do not have a glutamic acid residue at the -3 position, such as for example JAM-4 (Figure 18b).

The fact that the K499E mutation might affect the functionality of the PDZ1 domain towards some MAGI-1 binding proteins, prompted us to investigate whether this might impinge on the pattern of MAGI-1 sub-cellular localization, and in particular on its membrane targeting, since the integrity of the PDZ domains has been reported to be important for this function (Dobrosotskaya and James, 2000; Laura *et al.*, 2002). A previously identified membrane-bound interaction partner for MAGI-1 is β-catenin, and although the binding site for β-catenin is spatially segregated from the PDZ1 domain, interacting with the PDZ5 domain of MAGI-1 (Dobrosotskaya and James, 2000), we speculated that this association could be a marker to indirectly test the functionality of the K499E MAGI mutant. We repeated the co-immunoprecipitation experiments in 293 cells by overexpressing HA-tagged β-catenin alone or in combination with the FLAG-tagged M1wt or M1K499E constructs. After 24h cells were harvested and M1wt or the M1K499E were immunoprecipitated by incubating cell extracts with anti-FLAG conjugated agarose beads. Co-immunoprecipitated β-catenin was detected by western blot using anti-HA antibody, and the results of this assay are shown in Figure 19a. As can be seen, β-catenin co-immunoprecipitated with similar efficiencies with both the wild-type and mutant MAGI-1, suggesting that the membrane targeting of MAGI-1 is not affected by the K499E mutation. To confirm this, we also compared the expression pattern of wild-type and mutant MAGI-1 by immunofluorescence analysis. To do this FLAG-tagged M1wt and M1K499E were transfected into U2OS cells and, after 24 hours, the cells

were fixed and stained with anti-FLAG antibody. As can be seen from Figure 19b, and in agreement with previous studies (Dobrosotskaya and James, 2000), wild-type MAGI-1 displayed a differential pattern of sub-cellular localization, with different pools of MAGI-1 detectable in the nucleus as well as in the cytoplasm of transfected cells, whereas a prominent membrane staining was also apparent in cells forming intercellular junctions. In good agreement with the β-catenin co-immunoprecipitation experiments, the sub-cellular distribution of M1K499E was very similar to the wild-type protein, confirming that the K499E mutation does not affect the sub-cellular distribution of MAGI-1.

Taken together these data suggest that the K499E mutation which blocks HPV-16 and -18 E6 recognition, is only likely to affect the ability of MAGI-1 to interact with its cellular partners that recognize the PDZ1 domain and which have a glutamic acid residue at the -3 position of the PBM. Consistent with this, the K499E mutation does not appear to affect the sub-cellular localization of MAGI-1.

The M1K499E mutation potentiates the ability of MAGI-1 to establish tight junctions in HeLa cells

Previous studies defined an important role for MAGI-1 in the establishment of TJs, by promoting the recruitment of TJ-associated proteins such as ZO-1 and occludin (Hirabayashi *et al.*, 2003), and recently the silencing of HPV-18 E6 in HeLa cells was shown to promote the junctional accumulation of ZO-1 through MAGI-1 (Figure 14; Kranjec and Banks, 2011). These observations, prompted us to investigate whether the expression of MAGI-1 was sufficient to promote the junctional recruitment of TJ-associated proteins in HeLa cells, and to define whether the K499E mutant could potentiate this activity of MAGI-1. To do this, we decided to monitor the expression pattern of the TJ-associated proteins ZO-1 and PAR3 (Stevenson *et al.*, 1986; Izumi *et al.*, 1998; Kranjec and Banks, 2011). HeLa cells were grown on glass coverslips and transfected with the FLAG-tagged MAGI-1 constructs; 24 hours after transfection cells were fixed and the expression patterns of MAGI-1, ZO-1 and PAR3 were analysed by confocal microscopy. In order to evaluate whether the expression of M1wt and M1K499E could confer an advantage in terms of junctional

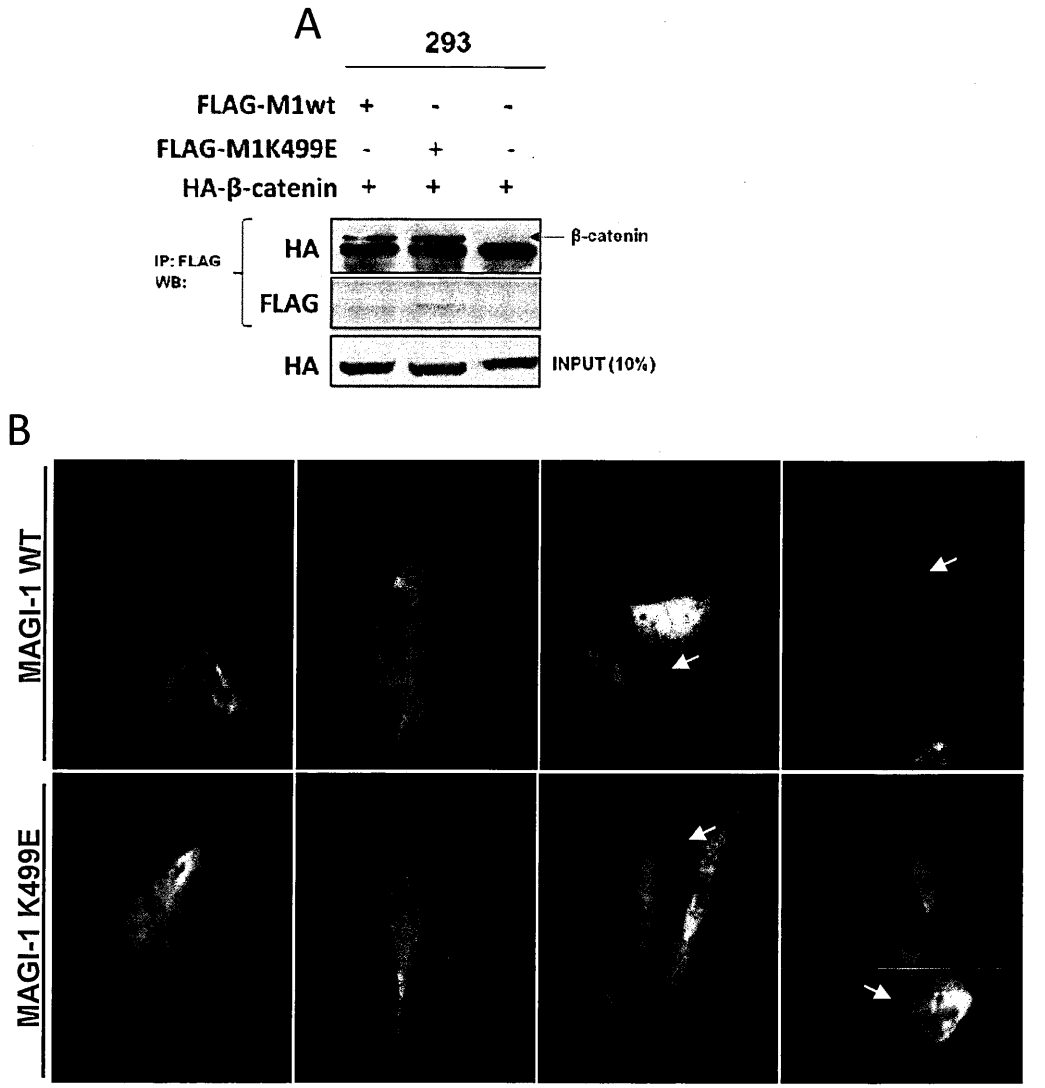

Figure 19. The K499E mutation does not affect the interaction of MAGI-1 with β-catenin and does not affect its localization. A. HEK293 cells were transfected with 3μg of wild-type or mutant MAGI-1 expression plasmids together with 5μg of HA-tagged β-catenin. After 24 hours cell extracts were immunoprecipitated using anti FLAG-antibody conjugated agarose beads, and MAGI-1-bound β-catenin was then detected by western blot using anti-HA antibody. The arrow indicates the position of HA-tagged β-catenin. B. U2OS cells were transfected with the FLAG-tagged MAGI-1 constructs, and after 24 hours the cells were fixed and incubated with anti-FLAG antibody to detect the MAGI-1 pattern of expression. White arrows indicate sites of MAGI-1 junctional accumulation.

assembly, multiple confocal fields were used to count transfected and untransfected HeLa cells until at least 100 FLAG-positive cells were analysed. Within each sample, HeLa cells were divided into subpopulations based on their positivity for the FLAG staining (MAGI-1 transfected or untransfected) and on their ability to form ZO-1 and PAR3-positive cellular junctions. As can be seen in Figure 20a, the vast majority of HeLa cells did not display junctional staining of ZO-1, and a similar pattern of staining was also observed for PAR3 (Figure 20b), suggesting that that HeLa cells are largely unable to form intact cellular junctions. This was also confirmed by direct cell counting, since within the untransfected subpopulation about 10-20% of HeLa cells displayed ZO-1- and PAR3-positive junctional staining (Figure21a). Strikingly, the expression of wild type and mutant MAGI-1 significantly increased the junctional assembly in HeLa cells, with the wild type and K499E mutant MAGI-1 proteins displaying similar efficiencies in promoting the junctional recruitment of ZO-1 and PAR3 (Figure 20 and Figure 21a). This is in agreement with the fact that the K499E mutation does not affect the ability of MAGI-1 to localize at junctional sites between contacting cells (Figure 18c), and further suggests that the mutant retains the capacity to restore junctional complexes. Furthermore, when compared with the wild type protein, the expression of the K499E mutant produced a much higher number of MAGI-1-positive cells, which is consistent with its increased resistance to E6-mediated degradation (Figure 20a and 20b, Figure 21b). In addition, this increased proportion of K499E MAGI-1-expressing cells was reflected in a corresponding increase in the number of cells showing junctional recruitment of ZO-1 and PAR3 (Figure 20a and 20b, Figure 21c).

Taken together, these data suggest that wild type and mutant MAGI proteins display comparable efficiencies in recruiting ZO-1 and PAR3 to cell contact sites, however the resistance of the K499E mutant to E6 degradation increases the number of HeLa cells with intact cell junctions, indicating that MAGI-1 expression potently enhances the ability of HeLa cells to form junctional complexes.

A well known biological effect associated with the establishment of junctional complexes is the inhibition of cell proliferation (Balda *et al.*, 2003; Aijaz *et al.*, 2005; Sottocornola *et al.*, 2010).

Previous studies indicated that both ZO-1 and PAR3 can inhibit cell proliferation when localized at cell contact sites by modulating the activity of multiple proteins implicated in G1/S cell cycle transition (Balda and Matter, 2000; Sottocornola *et al.*, 2010). Therefore, having shown that the expression of K499E mutant MAGI-1 can potently increase the junctional recruitment of ZO-1 and PAR3, we were interested in determining whether this could also correlate with an inhibition of cell proliferation in HeLa cells. In order to do this, we labeled proliferating cells with the thymidine analogue 5-ethynyl-2'-deoxyuridine (EdU), which allows the visualization of proliferating cells while not affecting the overall structural integrity of the cell (Salic and Mitchison, 2008). HeLa cells were grown on glass coverslips and transfected with the FLAG-tagged M1wt and M1K499E constructs. 24h post transfection, and prior to fixation, HeLa cells were pulsed with EdU for additional 2 hours, and the pattern of MAGI-1 and ZO-1 expression and the proportion of EdU-positive cells were visualized by confocal microscopy. As can be seen in Figure 22a, a high proportion of untransfected HeLa cells display positive nuclear EdU staining, demonstrating that the HeLa cells are highly proliferative. The expression of wild type MAGI-1 led to an increase in junctional ZO-1 staining and this correlated also with the absence of EdU staining in the M1wt-expressing cells. Conversely, K499E mutant MAGI-1, which also showed high levels of ZO-1 junctional recruitment, displayed a reduced ability to block the proliferation in HeLa cells compared with the wild type protein, suggesting that the integrity of MAGI-1 PDZ1 domain increases its ability to inhibit cell proliferation. The quantification of the number of EdU-positive nuclei relative to MAGI-1 expression, confirmed that wild type MAGI-1 dramatically reduced the proliferative potential of HeLa cells, and that the K499E mutation decreases the ability of MAGI-1 to inhibit cell proliferation by about 20% (Figure 22b). Nevertheless, consistent with the ability of mutant MAGI-1 to form larger MAGI-1-positive HeLa cell populations, its expression also correlates with an increased population of EdU-negative cells compared with the wild type protein (Figure 22c). These data suggest that MAGI-1 is a negative regulator of cell proliferation and that its expression in HeLa cells is capable of reducing the proportion of proliferating cells. However this regulation of the proliferative potential appears to be independent of MAGI-1's ability to

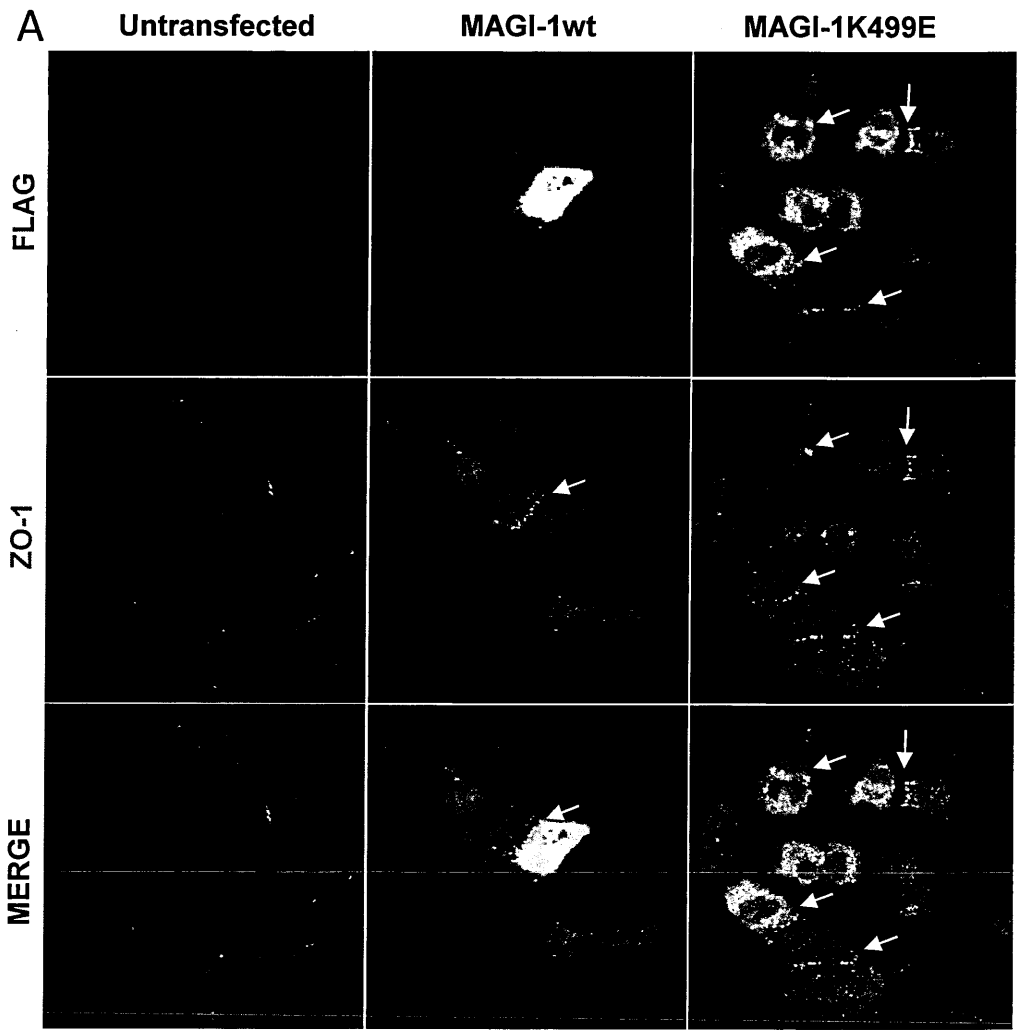

Figure 20. The expression of wild type and mutant MAGI-1 promotes junctional assembly in HeLa cells. A. HeLa cells were seeded on glass coverslips and transfected either with wild type or mutant MAGI-1. After 24 hours the cells were fixed and incubated with anti-FLAG and anti-ZO-1 antibodies and counterstained with rhodamine-conjugated (FLAG) and fluorescein-conjugated (ZO-1) secondary antibodies. Confocal images were taken at 480 and 510 nm wavelengths. Confocal images were taken at 480 and 510 nm wavelengths. White arrows indicate sites of MAGI-1 and ZO-1 junctional accumulation.

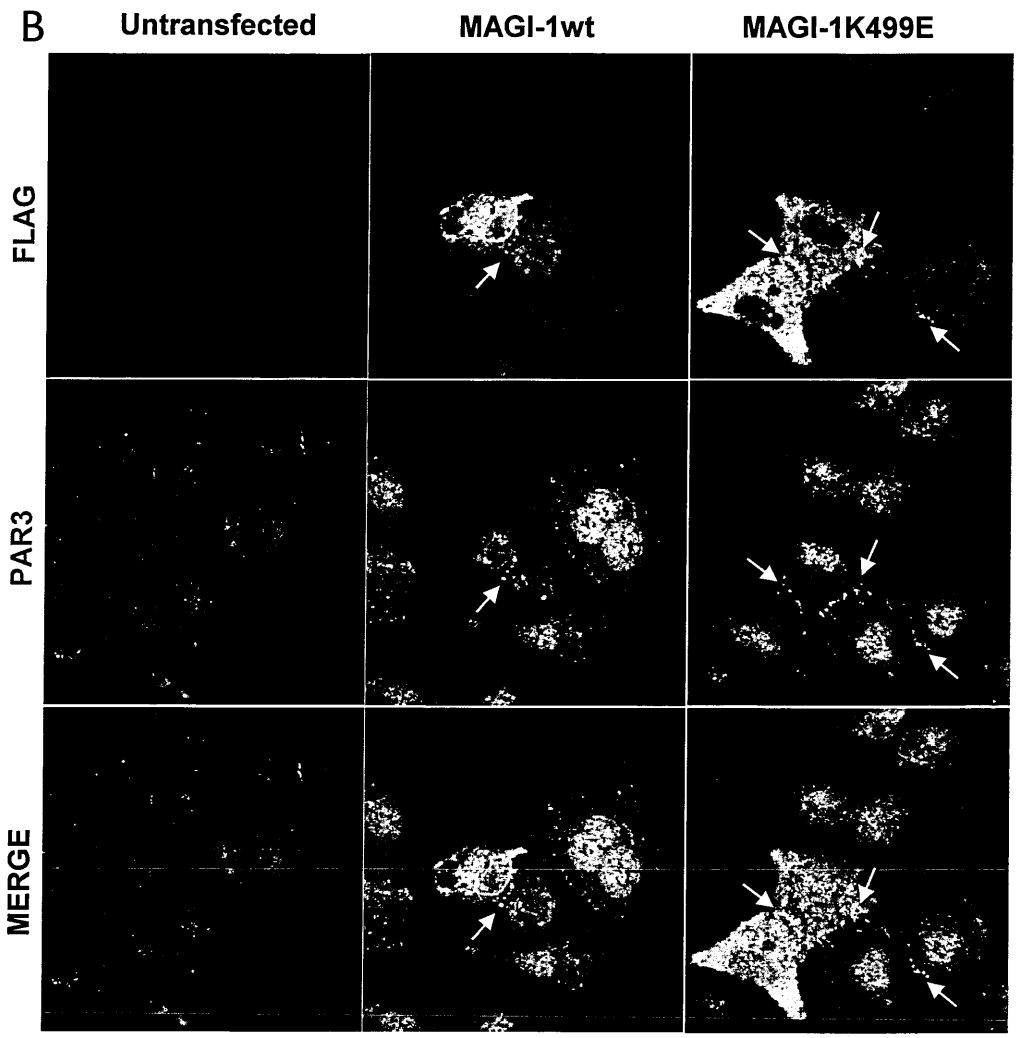

Figure 20 (Cont.). B. Cells were seeded and transfected as in A. After fixation, cells were incubated with anti-FLAG and anti-PAR3 antibodies and counterstained with rhodamine-conjugated (FLAG) and fluorescein-conjugated (PAR3) secondary antibodies. Confocal images were taken at 480 and 510 nm wavelengths. White arrows indicate sites of MAGI-1 and PAR3 junctional accumulation.

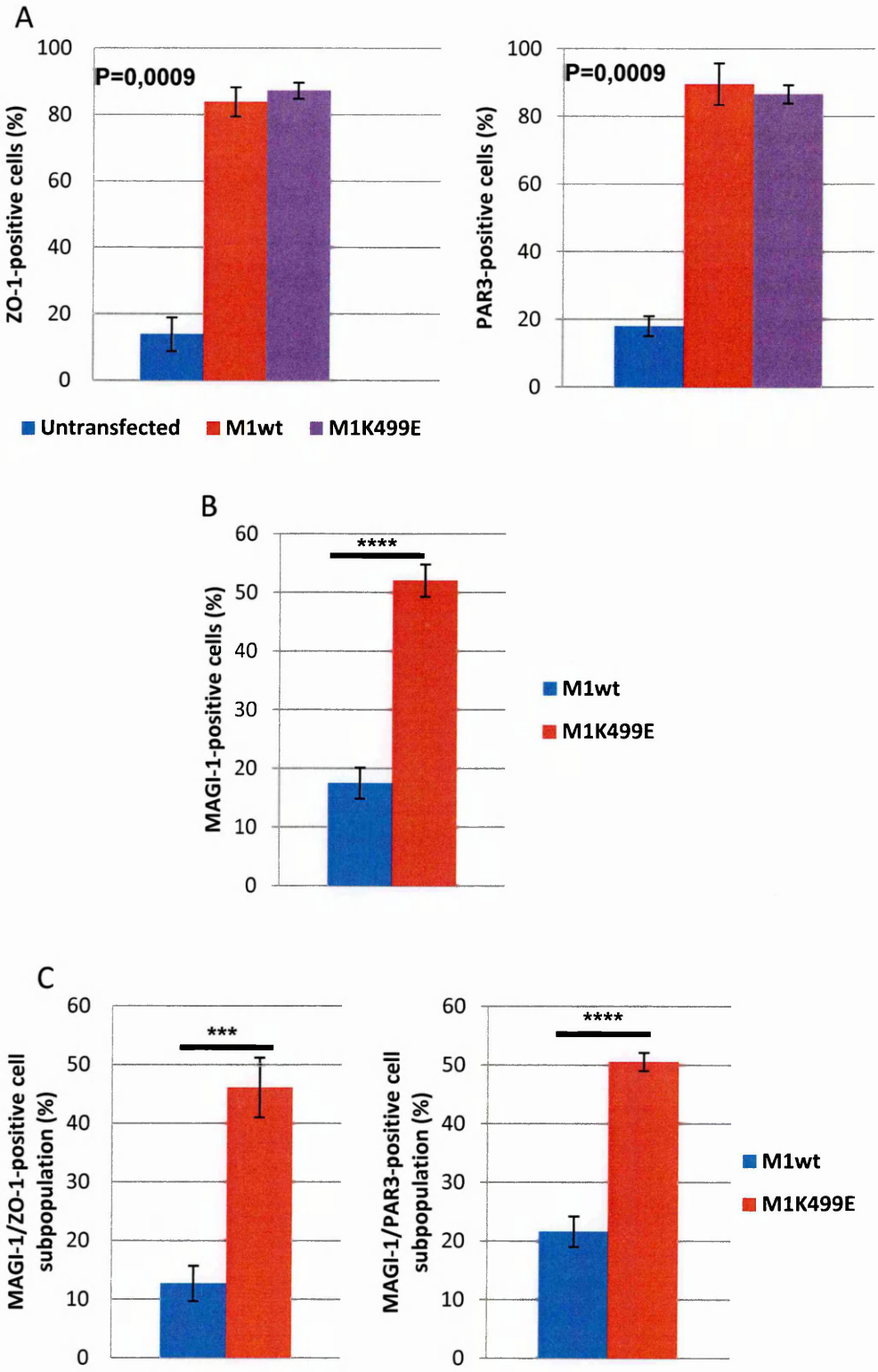

Figure 21. The resistance of MAGI-1 K499E to E6-mediated degradation correlates with an increased junctional assembly in HeLa cells. See the next page for the full legend.

Figure 21 (Cont.). The total number of HeLa cells showing M1- or M1K499E-positive staining were quantified within the total cell population visualized by immunofluorescence. In each experiment at least 100 wild type and mutant MAGI-1-positive cells were counted. A. The number of ZO-1 and PAR3-positive junctions formed in untransfected, wild type and mutant MAGI-1-transfected cells was calculated as the percentage of cells displaying ZO-1- and PAR3-positive junctional staining in each subpopulation (one way analysis of variance [ANOVA]). B. The total population of wild type and mutant MAGI-1-expressing cells was calculated as the percentage of FLAG-positive cells present in each sample relative to the total population of untransfected cells (P<0,0001; unpaired two-sample t-test). C. Percentage of MAGI-1-positive cells displaying ZO-1-positive (P=0,0006) and PAR3-positive (P<0,0001) junctional staining relative to the total population of untransfected cells counted in each sample (unpaired two-sample t-test). Each panel represents the mean values and standard deviations calculated from three independent experiments. The corresponding P values are: *P<0.05; **P<0.01; ***P<0.001;****P<0.0001.

restore TJs, since it seems to depend, in part, upon an as yet unidentified function of the MAGI-1 PDZ1 domain.

In order to assess whether the MAGI-1 is able also to mediate the inhibition of cell proliferation in HPV-negative cells, we repeated the EdU staining upon transfection of wild type and K499E mutant MAGI-1 in HaCaT cells, and the results are shown in Figure 23. As can be seen, in agreement with previous studies (Massimi *et al.*, 2012), HaCaT cells form cellular junctions between adjacent cells, as evidenced by junctional recruitment of ZO-1 (Figure 23a). Consistent with the data obtained in HeLa cells, wild type and K499E mutant MAGI-1 proteins co-localized with ZO-1 at cellular junctions, although the expression of either construct marginally increased the ZO-1 junctional recruitment. When the two MAGI-1 constructs were expressed in HaCaT cells, the effects on cell proliferation were markedly reduced when compared to HeLa cells (Figure 23a). This was highlighted by the quantification of the cells displaying an EdU-positive nuclear staining (Figure 23b), where about the 40% of MAGI-1-expressing cells also displayed an EdU-positive staining. As expected, in the absence of E6, the two constructs displayed a similar transfection efficiency (Figure 23c), however, consistent with lower efficiency of MAGI-1 in inhibiting the proliferation of HaCaT cells, also the percentage of EdU-negative and MAGI-1-positive cells compared to the total cell population was likewise reduced compared to HeLa cells (Figure 23d).

Taken together these data indicate MAGI-1 has a strong capacity to induce the junctional recruitment of TJ-associated proteins, and that the ability of MAGI-1 to inhibit cell proliferation could be linked to the inhibition of specific functions associated to the expression of E6 and E7.

MAGI-1 is a pro-apoptotic protein

The epifluorescent examination of HeLa cells stained for wild type and mutant MAGI-1 suggested us a possible involvement of this protein in the regulation of apoptosis, since proportions of the MAGI-1-expressing cells had morphological features resembling those of cells undergoing apoptosis, including blebbing and rounding up of the cells (Taylor *et al.*, 2008). In our opinion this

was particularly interesting since HeLa cells have been shown to be resistant to different apoptotic stimuli (Assefa *et al.*, 1999; Eichholtz-Wirth and Sagan, 2000), and MAGI-1 had been previously implicated in the regulation of apoptosis (Ivanova *et al.*, 2007; Gregorc *et al.*, 2007); however its possible direct involvement in the induction of apoptosis has not been previously investigated. A well studied biochemical event associated with the induction of apoptosis is the fragmentation of genomic DNA (Williams *et al.*, 1974; Tian *et al.*, 1991) due to the activation of cellular nucleases (Wyllie, 1980; Nagata *et al.*, 1998). Therefore, in order to determine whether the expression of wild type and K499E mutant MAGI-1 could promote apoptosis of HeLa cells, we decided to monitor their pattern of DNA fragmentation by performing terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays. In addition, we also included HaCaT cells in the analysis, in order to determine whether MAGI-1 could also induce apoptosis in HPV-negative cells. To do that, HeLa and HaCaT cells were seeded on glass coverslips and transfected either with wild type or K499E mutant MAGI-1 constructs. Since the induction of DNA fragmentation is a rather late event during induction of apoptosis (Collins *et al.*, 1997), we decided to grow the cells for 48h after transfection. The cells were then fixed, and the pattern of wild type and K499E mutant MAGI-1 expression as well as the number of cells positive for the TUNEL reaction, were assessed by immunofluorescence and confocal microscopy. Results are shown in Figure 24 and, as can be seen, untransfected HeLa (Figure 24a) and HaCaT (Figure 24b) cells both showed a low level of apoptosis, as evidenced by the low proportion of TUNEL-positive cells. Strikingly, the expression of wild type and mutant MAGI-1 led to the appearance of MAGI-1/TUNEL-positive cell subpopulations in both cell lines, suggesting that the ability of MAGI-1 to induce apoptosis is not exclusive to HPV-positive cells. In addition, the expression of the K499E mutant in HeLa cells led to a higher number of MAGI-1-positive cells than the wild type protein, and this also correlated with an increased subpopulation of MAGI-1/TUNEL-positive cells. It is also interesting to note that in HaCaT cells the TUNEL positivity associated with the transfection of the MAGI-1 constructs was not linked to the acquisition of a clear apoptotic morphology as in the case of HeLa cells under the same experimental conditions. However, this is consistent with the fact that some cell types can acquire an apoptotic morphology rather slowly, although they are already undergoing

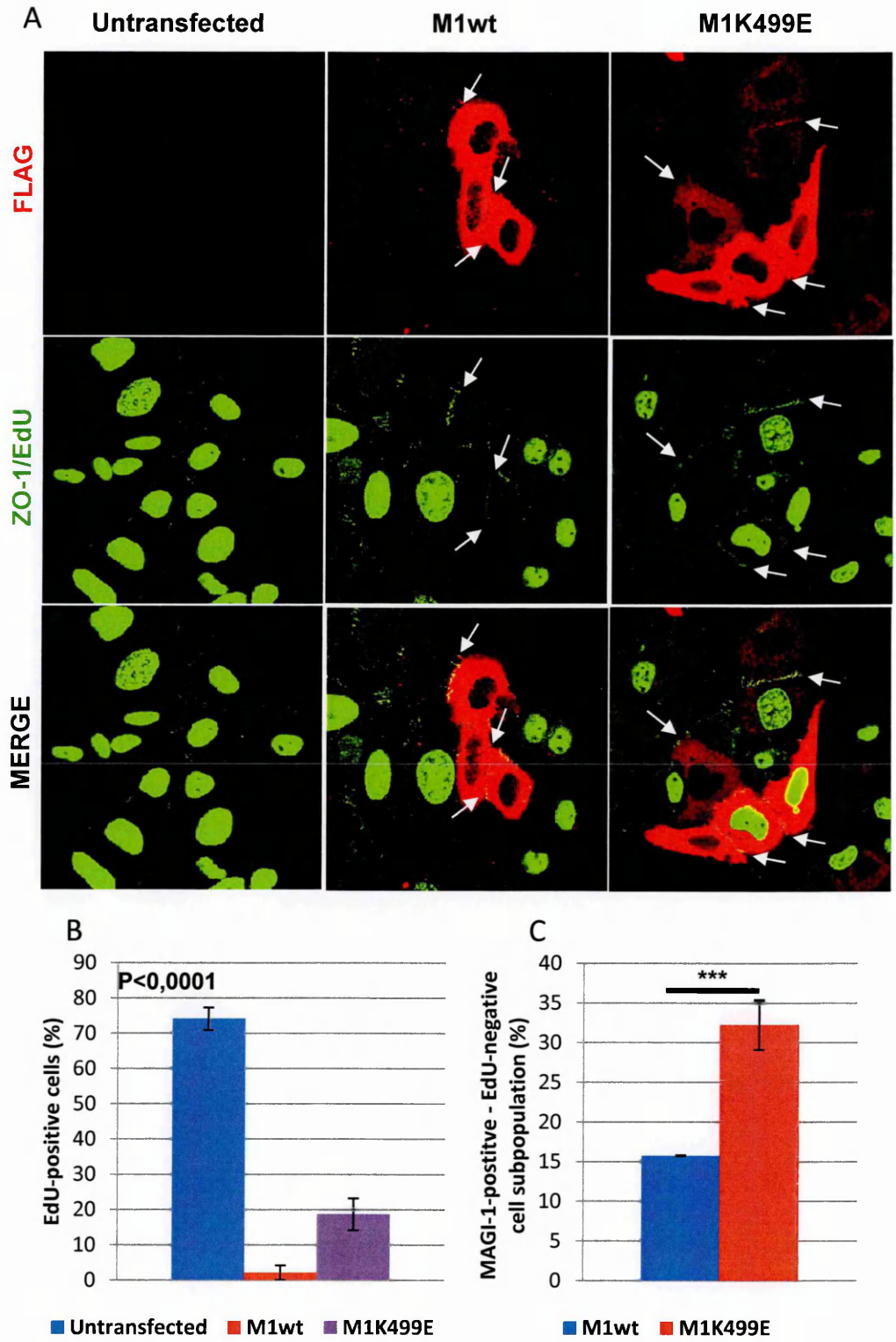

Figure 22. MAGI-1 inhibits cell proliferation of HeLa cells. See the next page for the full legend.

Figure 22 (Cont.). A. HeLa cells were seeded on glass coverslips and transfected either with FLAG-tagged wild type or mutant MAGI-1 constructs. After 24 hours, and prior to fixation, cells were incubated with EdU for additional 2 hours in order to allow its incorporation by proliferating cells. After fixation, cells were incubated with anti-FLAG and anti-ZO-1 antibodies and counterstained with rhodamine-conjugated (FLAG) and fluorescein-conjugated (ZO-1) secondary antibodies. After incubation with the indicated antibodies, cells were processed for the detection of EdU-labeled DNA (green nuclei). Confocal images were taken at 480 and 510 nm wavelengths. White arrows indicate sites of MAGI-1 and ZO-1 junctional accumulation. B. The percentage of EdU-positive cells was quantified by direct cell count of untransfected, wild type or K499E mutant MAGI-1-transfected cells displaying positive nuclear EdU staining within each subpopulation (one way analysis of variance [ANOVA]). C. Cells were counted as in B, and shown is the percentage of MAGI-1-positive cells displaying EdU-negative staining relative to the total population of untransfected cells counted in each sample (P=0,0008; unpaired two-sample t-test). Each panel represents the mean values and standard deviations calculated from three independent experiments. The corresponding P values are: *P<0.05; **P<0.01; ***P<0.001;****P<0.0001.

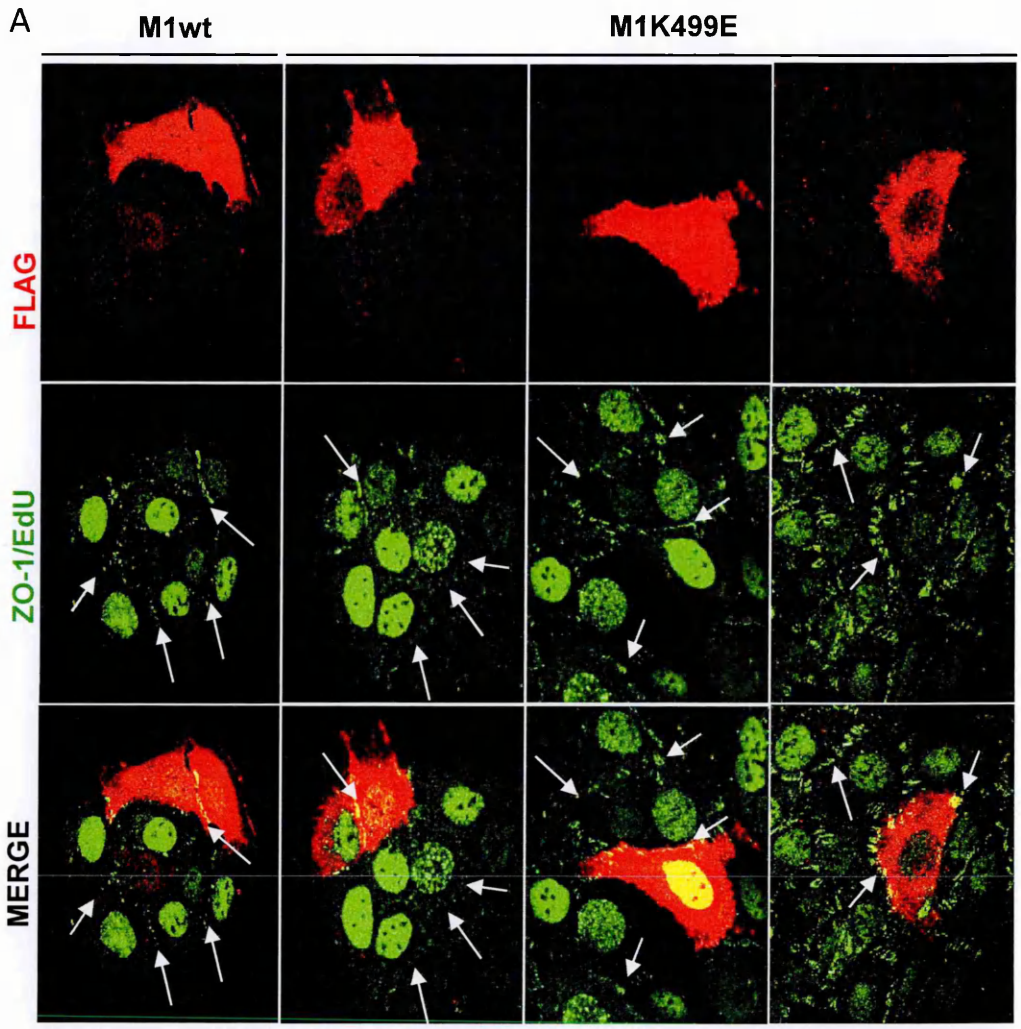

Figure 23. MAGI-1 is less efficient to inhibit the cell proliferation of HPV-negative cells. A. HaCaT cells were seeded on glass coverslips and transfection was carried out as in Figure 22. After 24 hours, and prior to fixation, cells were incubated with EdU for additional 2 hours in order to allow its incorporation by proliferating cells. After fixation, cells were incubated with anti-FLAG and anti-ZO-1 antibodies and counterstained with rhodamine-conjugated (FLAG) and fluorescein-conjugated (ZO-1) secondary antibodies. After incubation with the indicated antibodies, cells were processed for the detection of EdU-labeled DNA (green nuclei). Confocal images were taken at 480 and 510 nm wavelengths. White arrows indicate sites of MAGI-1 and ZO-1 junctional accumulation.

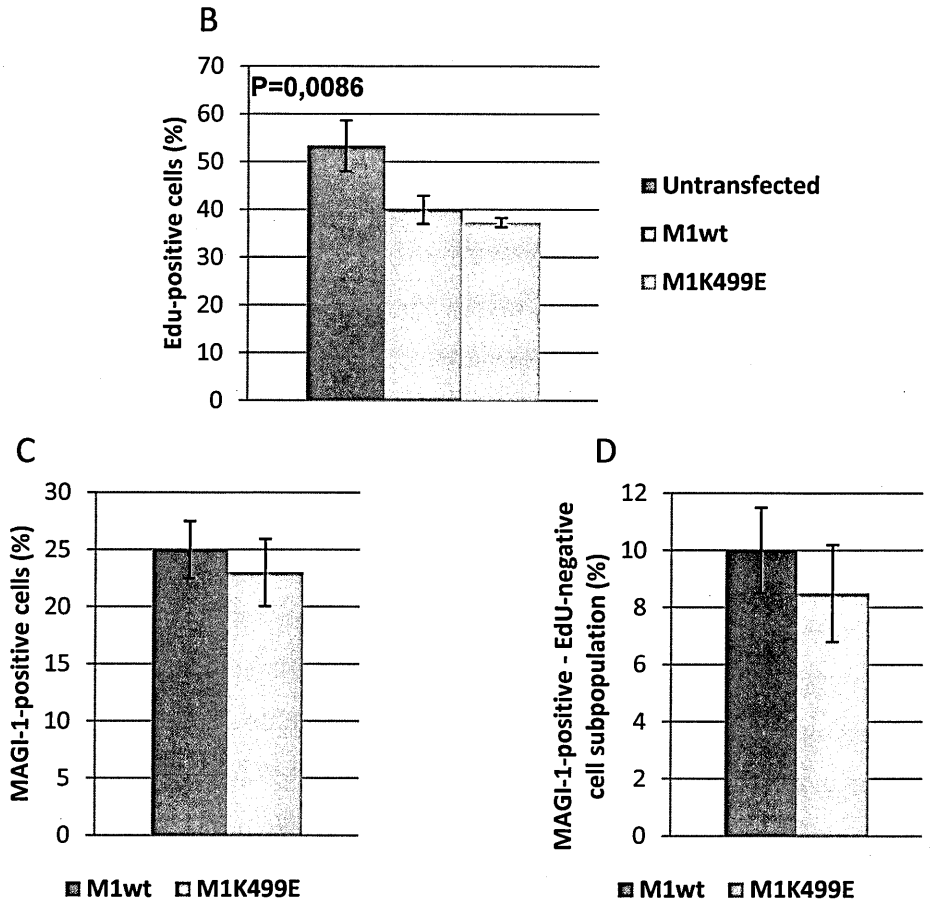

Figure 23 (Cont.). B. The percentage of EdU-positive cells was quantified by direct cell count of untransfected, wild type or K499E mutant MAGI-1-transfected cells displaying positive nuclear EdU staining within each subpopulation (one way analysis of variance [ANOVA]). C. The total population of wild type and mutant MAGI-1-expressing cells was calculated as the percentage of FLAG-positive cells (~100 cells per sample) present in each sample relative to the total population of untransfected cells. D. Cells were counted as in B, and shown is the percentage of MAGI-1-positive cells displaying EdU-negative staining relative to the total population of untransfected cells counted in each sample. Each panel represents the mean values and standard deviations calculated from three independent experiments. The corresponding P values are: *P<0.05; **P<0.01; ***P<0.001;****P<0.0001.

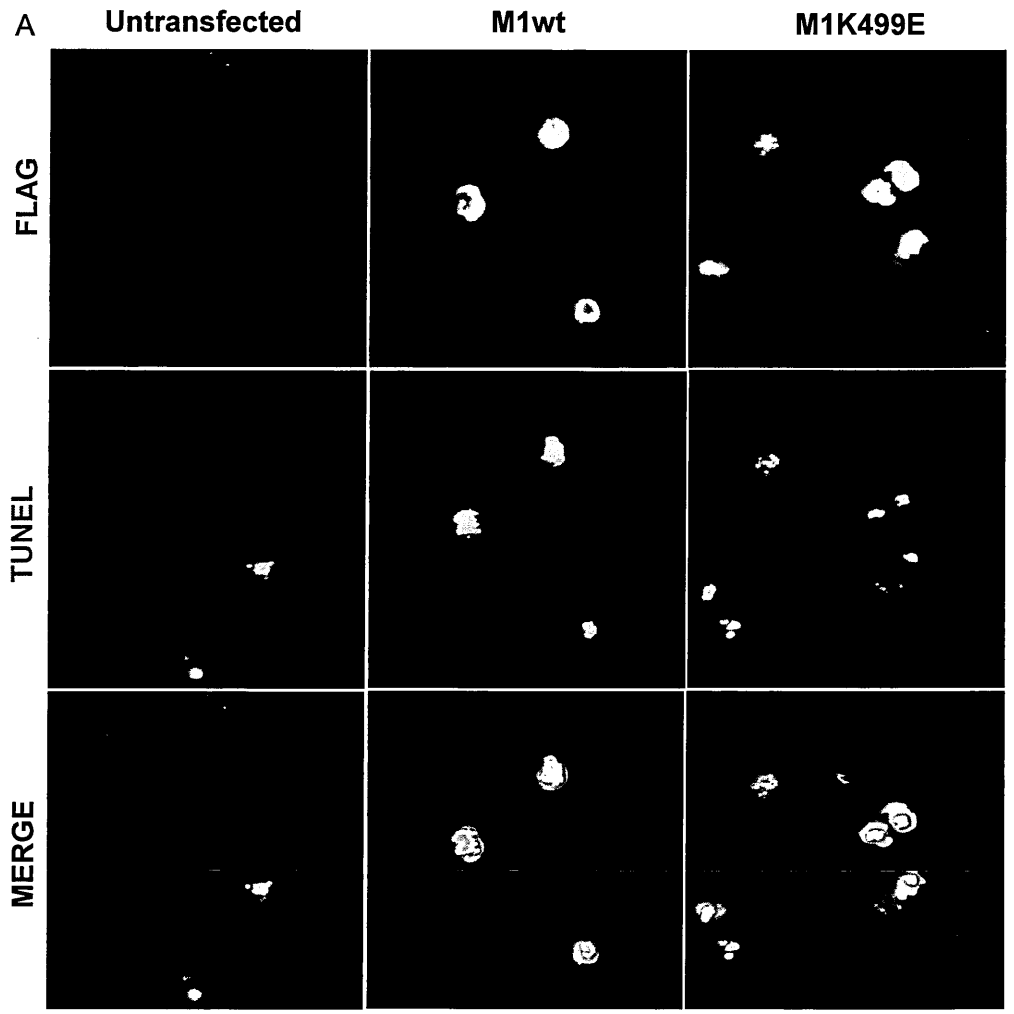

Figure 24. The expression of MAGI-1 induces apoptosis in HeLa and HaCaT cells. A. HeLa cells were seeded on glass coverslips and transfected either with wild type or mutant MAGI-1. After 48 hours the cells were fixed and incubated with the anti-FLAG antibody and counterstained with rhodamine-conjugated secondary antibodies. After incubation with the indicated antibodies, cells were processed for the identification of TUNEL-positive cells using the In situ cell death detection kit according to the manufacturer's instructions. Confocal images were taken at 480 and 510 nm wavelengths.

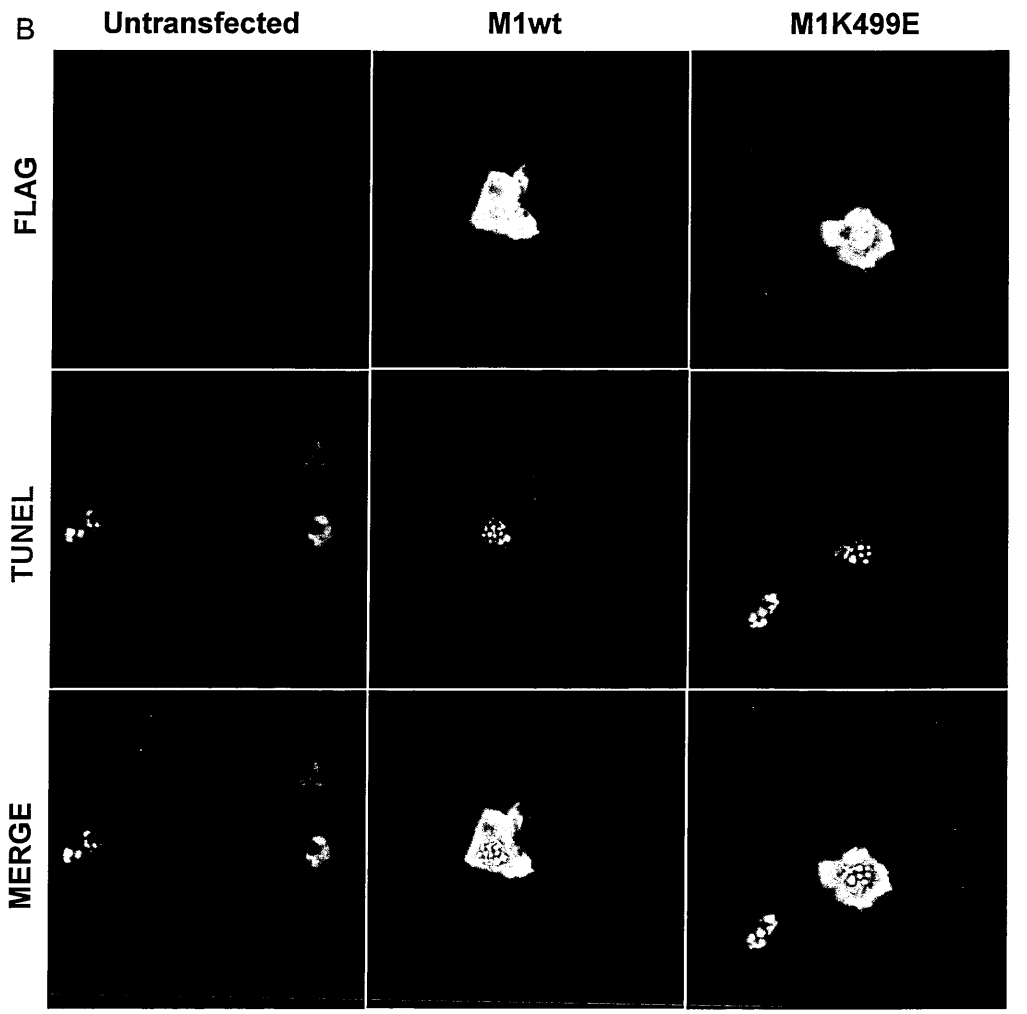

Figure 24 (Cont.). B. HaCaT cells were seeded on glass coverslips, and the immunodetection of MAGI-1 and the TUNEL assay were performed as in A.

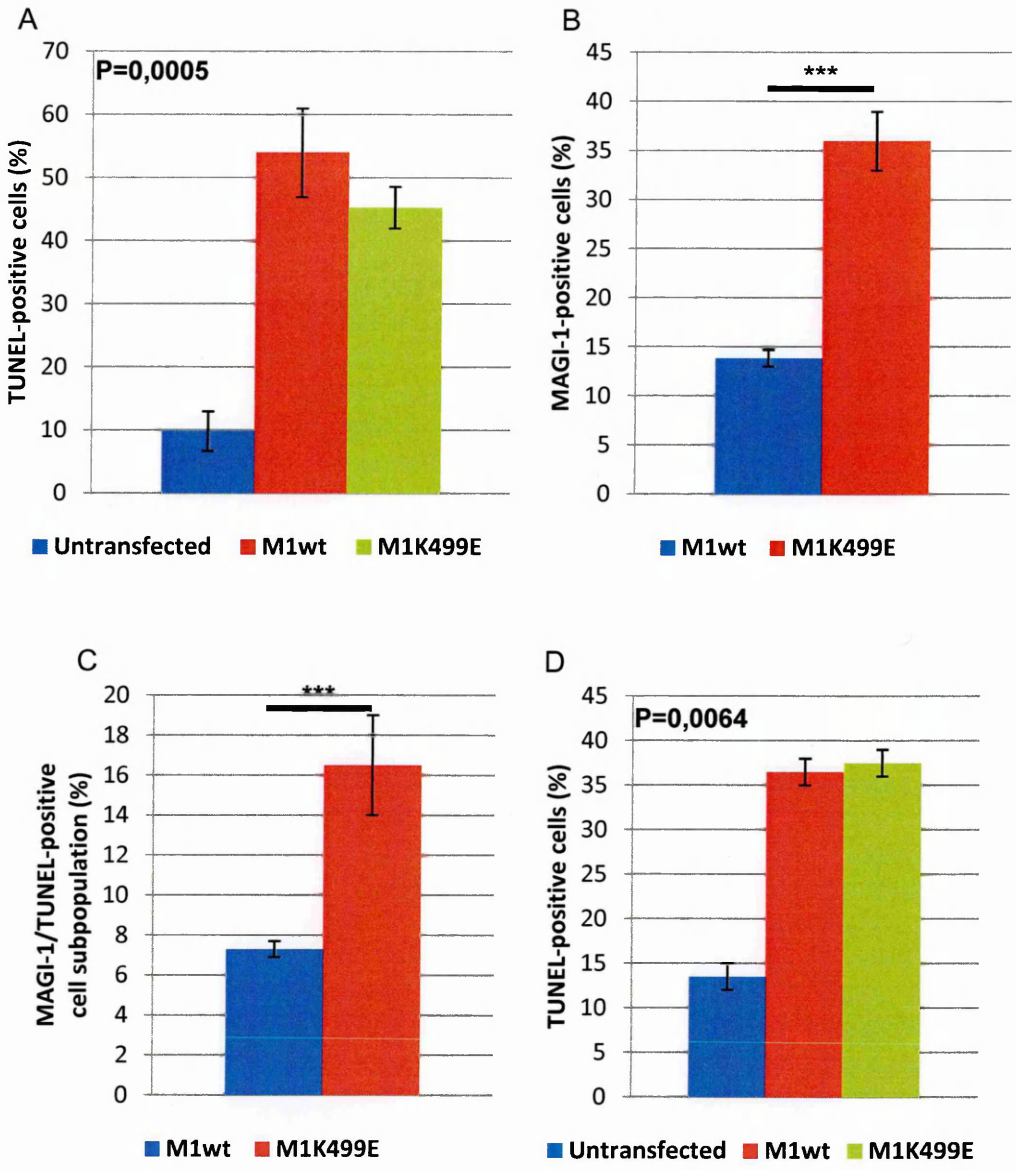

Figure 25. The expression of MAGI-1 correlates with induction of apoptosis. See the next page for the complete legend.

Figure 25 (Cont.). The expression of MAGI-1 correlates with induction of apoptosis. A. The percentage of TUNEL-positive HeLa cells was quantified by direct cell count of untransfected, wild type or K499E mutant MAGI-1-transfected cells displaying positive TUNEL staining within each subpopulation (one way analysis of variance [ANOVA]). B. The total population of wild type and mutant MAGI-1-expressing cells was calculated as the percentage of FLAG-positive cells (~100 cells per sample) present in each sample relative to the total population of untransfected cells (P=0,0002; unpaired two-sample t-test). C. Cells were counted as in A and B, and shown is the percentage of MAGI-1-positive cells displaying TUNEL-positive staining relative to the total population of untransfected cells counted in each sample (P=0,0033; unpaired two-sample t-test). D. The percentage of wild type or K499E mutant MAGI-1-transfected HaCaT cells displaying positive TUNEL staining was determined as in A (one way analysis of variance [ANOVA]). Each panel represents the mean values and standard deviations calculated from three independent experiments. The corresponding P values are: *P<0.05; **P<0.01; ***P<0.001;****P<0.0001.

the biochemical events associated with the apoptosis (Oberhammer *et al.*, 1994), in addition this also suggests that the kinetics of the induction of apoptosis are different between HeLa and HaCaT cells. Therefore, these data indicate that the increased resistance of K499E mutant MAGI-1 to E6-mediated degradation also correlates with an increased induction of apoptosis. In order to gain more quantitative insights about the ability of MAGI-1 to induce apoptosis, we decided to count the cells in each sample and to correlate the number of TUNEL-positive cells to the status of MAGI-1 expression. At least 100 cells were counted per sample, and as can be seen in Figure 25a, and in good agreement with the confocal images, only a small proportion of untransfected HeLa cells displayed TUNEL-positive staining; in contrast the expression of either wild type or mutant MAGI-1 led to a strong induction of apoptosis. It is also interesting to note that the two MAGI-1 proteins displayed a similar efficiency in inducing apoptosis, suggesting that the PDZ domain 1 is probably not involved in this function of MAGI-1. In addition, the expression of the K499E mutant MAGI-1 in HeLa cells generated a larger MAGI-1-positive population than the wild type protein (Figure 25b), and, consistent with this, also the MAGI-1/TUNEL-positive subpopulation was higher upon the expression of the mutant (Figure 25c). Untransfected HaCaT cells showed a slightly higher susceptibility to apoptosis induction compared to untransfected HeLa cells, however the expression of the MAGI-1 constructs also induced a high degree of apoptosis but with similar efficiencies (Figure 25d), although the levels in HaCaT were slightly lower then in HeLa.

Taken together, these data suggest that the expression of MAGI-1 increases the rate of cell death in both HPV-positive and HPV-negative cells, and that this function of MAGI-1 is likely not to be exerted through the PDZ domain 1. Moreover, this provides an additional advantage for HPV to promote the E6-mediated degradation of MAGI-1.

Part II:

Regulation of HPV-18 E6 expression by hScrib

hScrib specifically stabilizes HPV-18E6 protein levels

It is well established that the continuous expression of E6 and E7 oncoproteins is a prerequisite for maintaining the survival and tumorigenic potential of HPV-positive cancer cells. Recent studies suggested that the interaction of high-risk HPV E6 oncoproteins with PDZ domain-containing proteins could be beneficial for the viral life cycle by maintaining high levels of E6 expression (Nicolaides *et al.*, 2011). Furthermore, these studies also suggested that this phenomenon was linked to the direct stabilization of E6 by several different PDZ substrates rather than by one specific E6 target. However, all of these analyses were done under conditions of ectopic overexpression, and we were interested in determining whether different PDZ domain-containing substrates of E6 could likewise contribute to maintaining the steady state levels of E6 in cells derived from cervical lesions, in which E6 is expressed at endogenous levels. To do this, we decided to investigate the effect of siRNA ablation of several different PDZ domain-containing targets of E6 upon the steady state levels of E6 expression in HeLa cells, as reagents for the detection of HPV-18 E6 are readily available (Tomaić *et al.*, 2009; Krishna Subbaiah *et al.*, 2012). Cells were transfected with siRNA against Luciferase, hScrib, hDlg, TIP2, PSD95, MAGI-1, PTPN3 and FAP1. We also included in the analysis siRNA against HPV-18 E6/E7 as a reference for E6 downregulation. 72 hours after transfection, the cells were extracted and the expression levels of HPV-18 E6, p53 and the set of silenced PDZ proteins were monitored by western blot analysis. In addition, since recent studies had shown that the stability of HPV-18 E6 in HeLa cells is dependent upon E6AP expression (Tomaić *et al.*, 2009), we also assessed the levels of E6AP in order to determine whether PDZ proteins might affect the expression of E6 indirectly through the modulation of E6AP levels. The results are shown in Figure 26a, and the quantifications of HPV-18 E6 levels from multiple experiments are shown in Figure 26b. As can be seen, control siLuciferase HeLa cells express readily detectable levels of endogenous HPV-18 E6, p53 and E6AP as well as of the PDZ proteins TIP2 and hScrib, whereas lower residual levels of hDlg

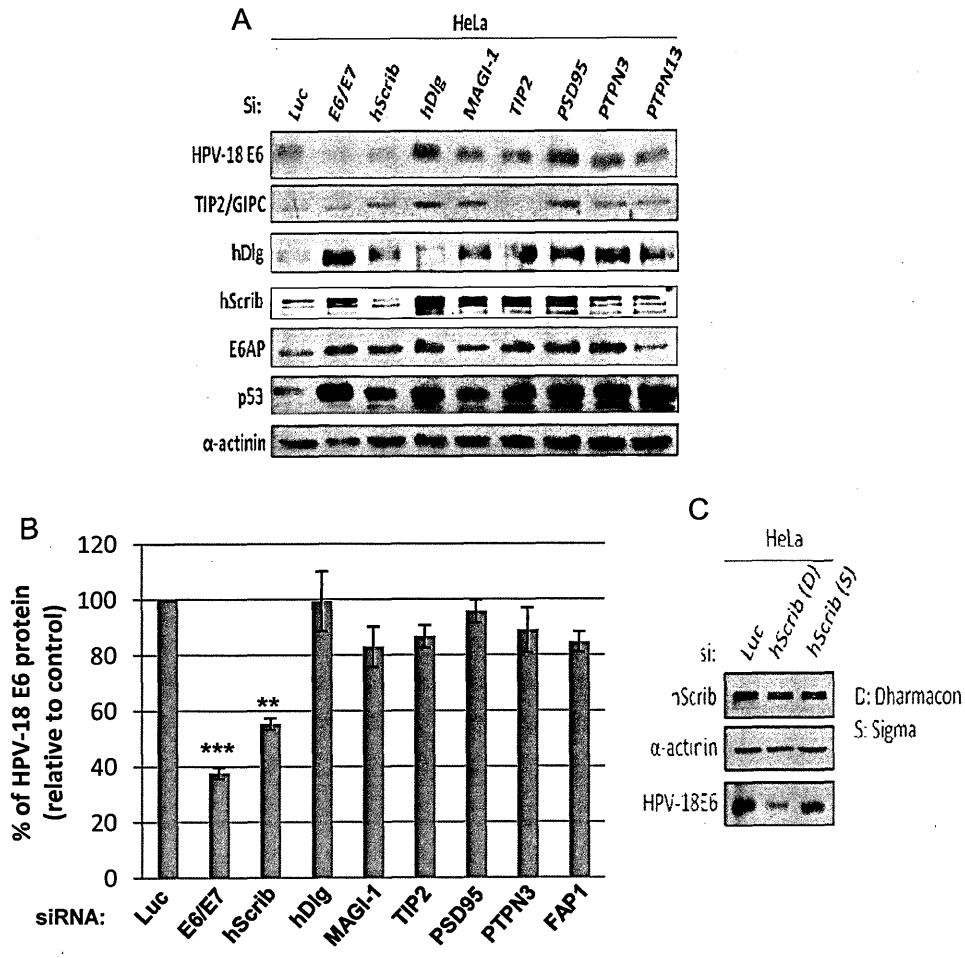

Figure 26. hScrib regulates the expression of HPV-18 E6 in HeLa cells.

Figure 26 (Cont.). A. HPV-positive HeLa cells were transfected with siRNA Luciferase, siRNA HPV-18 E6 or siRNA against the indicated E6 PDZ substrates. Cells were grown for 72 hours prior harvesting and the expression patterns of HPV-18 E6, hDlg, hScrib, TIP2, p53, E6AP and α-actinin, to monitor the protein loading, were assessed by western blot analysis. B. Band intensities were determined using the OptiQuant quantification program. E6 levels were normalized to 100% relative to siLuciferase-transfected HeLa cells. Histograms represent the mean values and standard deviations calculated from three independent experiments (unpaired two-sample t-test). C. The silencing of hScrib was performed as in A but using two different siRNAs specific for hScrib. The expression levels of HPV-18 E6, hScrib, p53 and α-actinin to monitor the protein loading, were assessed by western blot analysis. The corresponding P values are: *P<0.05; **P<0.01; ***P<0.001;****P<0.0001.

expression could be detected. Silencing of E6 and E7 produced a massive downregulation of HPV-18 E6 expression compared with control cells, and, as expected, this led to a significant increase of p53 expression levels, which is consistent with previous studies (Scheffner *et al.*, 1993; Huibregtse *et al.*, 1993), and with the results presented in Figures 10, 11 and 12. It is interesting to note that upon ablation of E6/E7 the expression levels of E6AP were also upregulated, and this is consistent with previous studies suggesting that high-risk HPV E6 promotes the self-ubiquitylation and increased rates of proteasome-mediated degradation of E6AP (Kao *et al.*, 2000). In addition, in agreement with previous results (Kranjec and Banks, 2011; Figure 10b and 10c), the silencing of HPV-18 oncoproteins in HeLa cells produced a rescue of hDlg levels of expression and also hScrib levels were increased. In contrast, TIP2 levels remained relatively unchanged. Surprisingly, among the E6 PDZ targets assayed, the ablation of hScrib produced a dramatic reduction of HPV-18 E6 levels of expression, with an efficiency that was slightly lower than that obtained with the E6/E7 siRNA (Figure 26a and 26b). Consistent with this reduction in E6 levels of expression, the ablation of hScrib also resulted in increased levels of hDlg, E6AP and p53, although to a lower extent than in siE6/E7 transfected HeLa cells. Interestingly, similar levels of E6AP upregulation were also obtained upon ablation of hDlg, TIP-2, PSD95 and FAP1, and similarly p53 expression was also generally increased upon the silencing of the other E6 PDZ targets, although in this case HPV-18 E6 levels were not significantly reduced. In this context, the modulation of p53 expression has been linked so far only to the PDZ protein TIP1, which by promoting increased p53 ubiquitylation, increases its levels of proteasome-mediated degradation (Han *et al.*, 2012), therefore loss of TIP1 would be expected to increase p53 levels. However, our data also suggest that p53 expression could, at least in part, be regulated by multiple PDZ domain-containing proteins in HeLa cells. In addition, silencing of hDlg also produced a strong upregulation in the levels of hScrib expression, which was even higher than that obtained following ablation of E6. Low levels of hScrib upregulation were also obtained by silencing MAGI-1, TIP2 and PSD95. Similarly, hDlg and TIP2 expression levels were also elevated upon silencing of the other PDZ domain-containing proteins, possibly suggesting the existence of a high level of compensation between different PDZ proteins.

In order to confirm that the maintenance of HPV-18 E6 levels in HeLa cells were indeed dependent upon the expression of hScrib, and to verify that this was not due to any off-target effects of the hScrib-specific siRNA we repeated the analysis with an alternative siRNA obtained from a different supplier. As can be seen in Figure 26c, the two hScrib siRNAs blocked the expression of hScrib, although with slightly different efficiencies. Consistent with the results in Figure 26a and 26b, the two hScrib siRNA produced a similar reduction in the HPV-18 E6 levels of expression, and this reduction correlated directly with the efficiency with which hScrib levels were reduced.

Taken together these data confirm that loss of hScrib expression in HeLa cells can directly lead to a reduction in the levels of E6 in HeLa cells, and that multiple PDZ proteins are involved in complex molecular networks that might regulate the function of p53 as well as of other PDZ proteins.

Previous studies suggested that HPV-18 E6 displays a differential subcellular localization, with protein pools localizing predominantly at membrane and nuclear sites of transfected cells (Grossman *et al.*, 1989). In order to investigate whether loss of hScrib affected the pattern of expression of specific pools of E6 we performed fractionation experiments on HeLa cells transfected with either siRNA against Luciferase or hScrib. 72 hours post-transfection, HeLa cells were separated into four subcellular fractions and the expression pattern of HPV-18 E6, p53 and of the fraction-specific markers α-tubulin, E-cadherin, p84 and vimentin were assessed by western blot analysis. As can be seen from Figure 27, in siLuciferase transfected cells we detected the majority of E6 in the membrane fraction, with lower levels present in the nuclear and cytoplasmic fractions. In cells transfected with control siRNA, p53 was mainly expressed in the nucleus with residual levels also found in membrane and cytoskeletal fractions. Conversely, in cells transfected with hScrib siRNA, the levels of E6 are generally reduced in all three fractions. Consistent with this, p53 levels increase slightly in the same three fractions. These data suggest that silencing of hScrib in HeLa cells affects the total levels of E6 expression, rather than a specific subcellular pool.

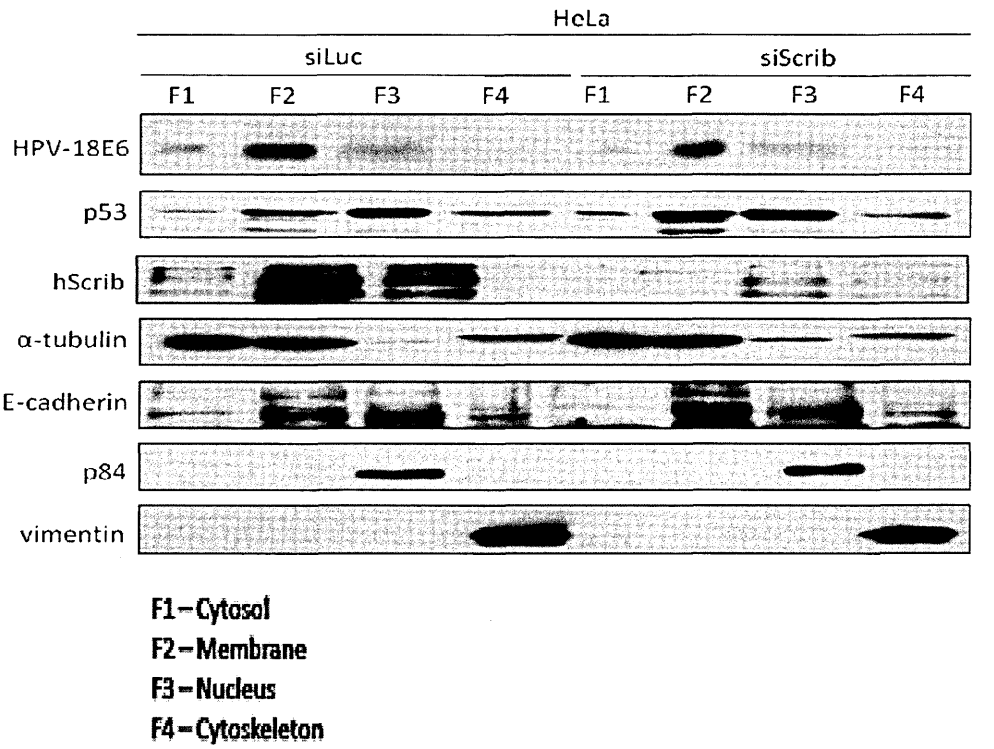

Figure 27. Loss of hScrib does not affect specific subcellular pools of E6 in HeLa cells. HeLa cells were transfected with siRNA Luciferase or siRNA hScrib, and after 72h cells were fractionated into 4 subcellular compartments: cytosol (F1), membrane (F2), nucleus (F3), cytoskeleton (F4). The expression patterns of HPV-18 E6, p53, hScrib and those of the four subcellular fraction markers E-cadherin (membrane), p84 (nucelus), α-tubulin (cytoplasm) and vimentin (cytoskeleton) were assessed by western blot analysis.

Ablation of hScrib does not affect HPV-18 E6 turnover

Having shown that loss of hScrib results in decreased levels of HPV-18 E6, we were interested in investigating whether this was due to an increase in the rate of E6 turnover, since previous studies had shown that the lack of PDZ binding capacity affected E6 stability (Nicolaides *et al.*, 2011). To do this, we decided to monitor the E6 half-life in HeLa cells following ablation of hScrib expression. 72 hours after transfection, the cells were treated with cycloheximide for different time-points. The cells were then harvested and the expression levels of E6 were monitored by western blotting. The results are shown in Figure 28a, with the quantifications from multiple experiments shown in Figure 28b. In agreement with previous studies, the half-life of HPV-18 E6 in control siRNA-transfected HeLa cells was between 60 and 120 minutes (Grossman *et al.*, 1989; Tomaić *et al.*, 2009). This is also in agreement with previous studies showing that different subcellular pools of E6 display differences in their half-life, and that the membrane pool of the protein, the predominant form of E6 found in HeLa cells, has a half-life of about 2 hours (Grossman *et al.*, 1989). The silencing of hScrib however, did not produce any significant change in the E6 half-life, suggesting that loss of hScrib expression does not affect E6 turnover. It is interesting to note, however, that although the turnover of E6 was not affected in siScrib transfected HeLa cells, p53 was stabilized in these cells. Consistent with previous studies, in control siRNA transfected HeLa cells the half-life of p53 was between 15 and 30 minutes (Talin *et al.*, 1998). The ablation of hScrib significantly prolonged the half-life of p53, which is consistent with the overall decrease in E6 levels, but this is not as a result of increased E6 turnover.

We were then interested in investigating whether hScrib might alter the levels of E6 gene expression. Since E6 and E7 are expressed from a bicistronic mRNA (Schneider-Gädicke and Schwarz, 1986; Smotkin and Wettstein, 1986), we reasoned that any reduction in E6 transcription in HeLa cells, would also be reflected by lower levels of E7 expression. The high-risk HPV E7 oncoprotein promotes the proteasome-mediated degradation of hypophosphorylated, E2F-binding competent forms of pRB (Boyer *et al.*, 1996; Gonzalez *et al.*, 2001). Therefore, the analysis of the expression pattern of differentially phosphorylated forms of pRB in HPV-positive cells can be used

as a surrogate marker to monitor the levels of E7 expression (Tang *et al.*, 2006). HeLa cells were transfected with control siLuciferase, siRNA against hScrib or HPV-18 E6/E7 as a reference for the silencing of E7. After 72 hours, cells were harvested and the expression pattern of pRB was monitored by western blot analysis. The results of this assay are presented in Figure 28c, and the quantifications of pRB levels obtained in multiple experiments are shown in Figure 28d. In agreement with previous studies, our results demonstrate that pRb is expressed as differentially phosphorylated forms, and that HeLa cells predominantly express the hyper-phosphorylated pRB (Tang *et al.*, 2006). Following ablation of E6 and E7, the expression of p53 and the ratio between the hypo- and hyper-phosphorylated pRB levels are significantly increased, demonstrating the efficient silencing of both oncoproteins. In contrast, whilst loss of hScrib expression led to an increase in p53 levels, the ratio between the hypo- and hyper-phosphorylated forms of pRB was comparable to that of the control siRNA-transfected HeLa cells, indicating that upon loss of hScrib the levels of E7 expression remained unaffected. Interestingly however, loss of hScrib resulted in an overall reduction of both pRB forms. This might be a reflection of a decrease in E6 levels, as previous studies suggested that E6 was able to induce high levels of expression of both hypo- and hyper-phosphorylated pRB (Malanchi *et al.*, 2004). Taken together these data indicate that loss of hScrib does not directly affect the levels of transcription of E6 and E7 in HeLa cells.

Loss of hScrib decreases HPV-18 E6 translation

We were next interested in determining whether E6 rates of translation were affected by loss of hScrib. To do this, HeLa cells were first transfected with siLuciferase or siScrib, and after 72 hours the cells were treated for 6 hours with cycloheximide to block protein translation. Cells were then washed several times with PBS to remove the cycloheximide, and the recovery in the levels of HPV-18 E6 and p53 protein expression were monitored over time by western blot analysis. The results are shown in Figure 29a, and the quantifications of multiple experiments are shown in Figure 29b. As can be seen, the prolonged treatment with cycloheximide produced over an 80% decrease in the levels of E6 in both control and siScrib transfected HeLa cells. Even more apparent was the drop in p53 levels, which upon cycloheximide treatment became undetectable in both the

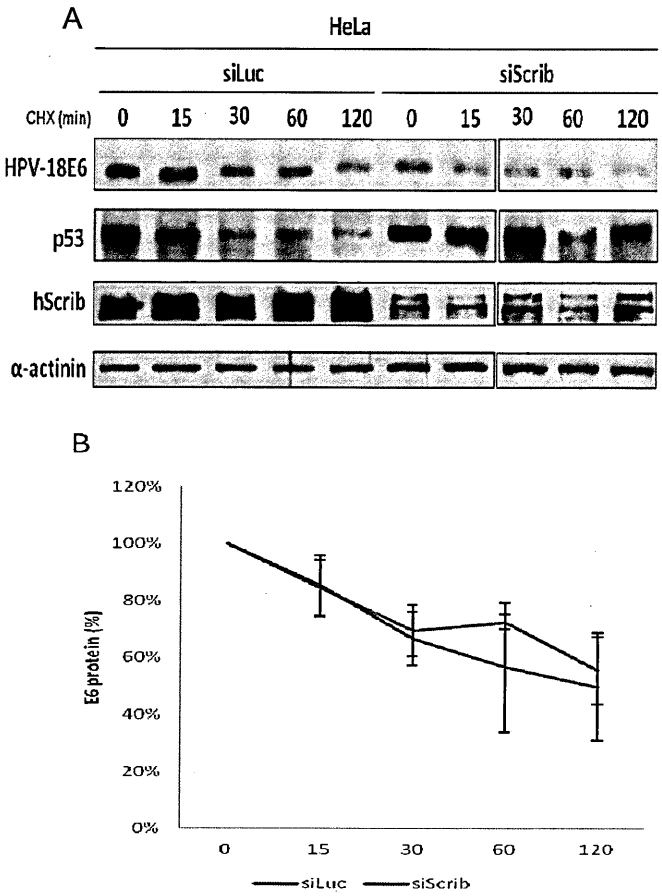

Figure 28. Loss of hScrib does not affect HPV-18 E6 protein stability nor its transcriptional rate. A. HeLa cells were transfected with siRNA against Luciferase or siRNA against hScrib, 72 hours after transfection and prior to harvesting, cells were treated with cycloheximide for 5 different time points: 0, 15, 30, 60 and 120 minutes. The expression levels of HPV-18 E6, p53, hScrib and α-actinin to monitor the protein loading, were assessed by western blot. The collated results from 3 independent experiments to measure E6 protein turnover in cells treated with siRNA Luciferase and siRNA hScrib are shown in panel B. Band intensities were determined using the OptiQuant quantification program. The E6 levels in siLuciferase and siScrib transfected cells were normalized to 100% at time 0. Shown are the mean values and standard deviations calculated from three independent experiments

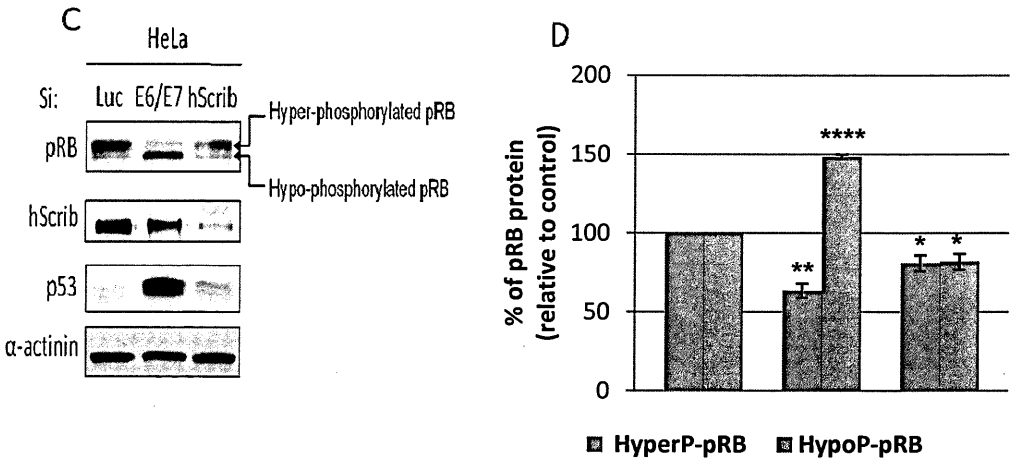

Figure 28 (Cont.). C. HeLa cells were transfected with siRNA against Luciferase or siRNA HPV-18 E6/E7 or siRNA hScrib. 72 hours after transfection, cells were harvested and the expression pattern of pRB, p53, hScrib and α-actinin, to monitor the protein loading, were assessed by western blot. D. Band intensities were quantified using the OptiQuant quantification program, and levels of of hypo-phosphorylated and hyper-phosphorylated pRB expression in control, siLuciferase-transfected cells, and siScrib-transfected cells were normalized to 100%. Differences in the expression pattern of pRB were expressed as percentage of hypo-phosphorylated and hyper-phosphorylated pRB protein relative to control cells. Histograms represent the mean values calculated from three independent experiments. Standard deviations are also shown (unpaired two-sample t-test). The corresponding P values are: *P<0.05; **P<0.01; ***P<0.001;****P<0.0001.

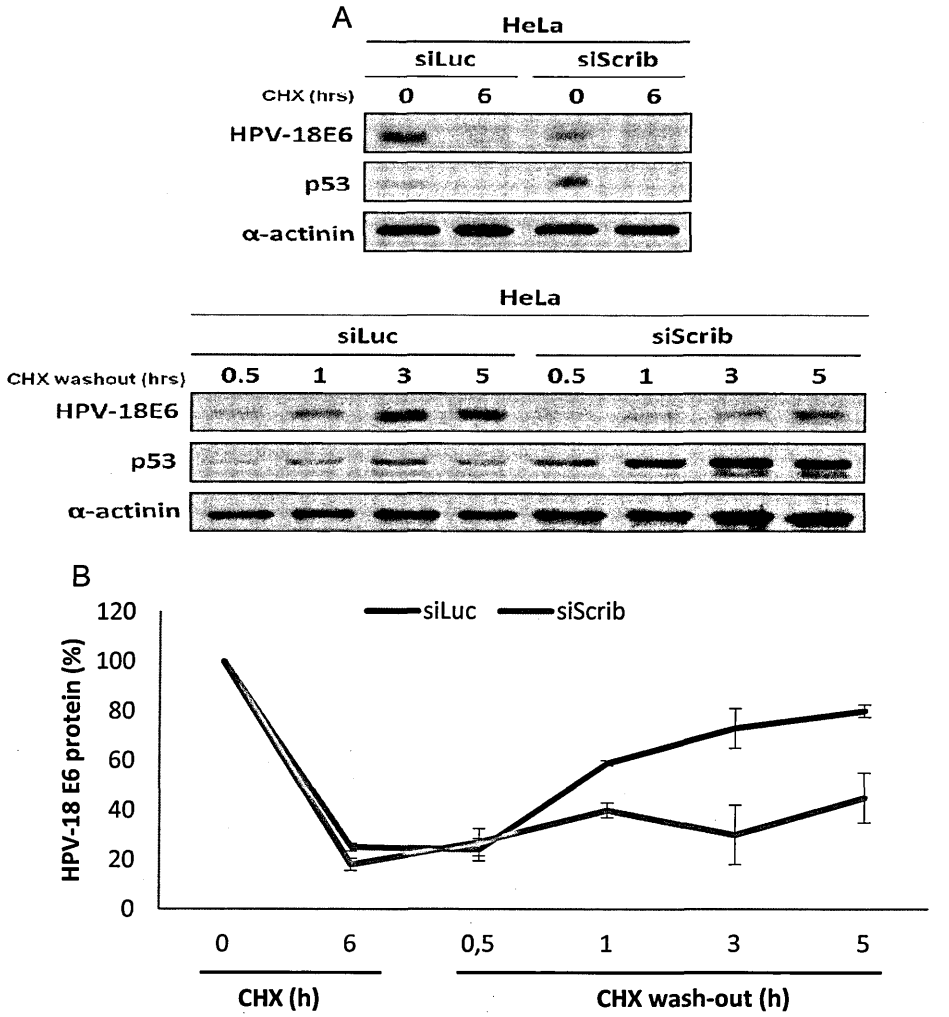

Figure 29. hScrib regulates the translation of HPV-18 E6 in HeLa cells. A. HeLa cells were transfected with siRNA against Luciferase or siRNA against hScrib. 72 hours after transfection, cells were treated with cycloheximide for an additional 6 hours. After the treatment, and prior to harvesting, cells were washed three times with PBS to remove the cycloheximide and protein translation was left to recover in complete medium for 4 different time points: 0.5, 1, 3 and 5 hours. The collated results from 3 independent experiments are shown in panel B. Band intensities were determined using the OptiQuant quantification program. The E6 levels in siLuciferase and siScrib transfected cells were normalized to 100% at time 0. Shown are the mean values and standard deviations calculated from three independent experiments.

control and siScrib transfected cells. Upon removal of cycloheximide, the levels of HPV-18 E6 expression progressively recovered after the 30 minute time point. It is interesting to note that the bulk of E6 oncoprotein was translated within 3 hours of cycloheximide wash-out, suggesting that the accumulation of E6-encoding mRNAs during translation inhibition led to the rapid synthesis of newly translated E6 upon removal of cycloheximide. In contrast, loss of hScrib in HeLa cells greatly reduced the rate of recovery in E6 protein levels upon translation re-initiation. Interestingly, the pattern of p53 recovery was opposite to that of E6, being rapidly upregulated in siScrib cells after cycloheximide wash-out, which is consistent with the lower levels of E6 expression in these cells. Taken together these data suggest that the residual expression of hScrib in HeLa cells contributes towards maintaining high levels of HPV-18 E6 expression through the modulation of its rate of translation.

hScrib regulates the mTORC1 pathway through the modulation of S6 kinase activity

Previous studies suggested that the E6 mRNA is translated using the canonical cap-dependent ribosome scanning model (Stacey *et al.*, 2000; Tan *et al.*, 1994). In this model, the 40S ribosome subunit contacts the 5' end of the mRNA and begins to scan the messenger until it recognizes a suitable start codon, at which the 60S ribosomal subunit is recruited to form the complete ribosome and begin the polypeptide translation (Kozak *et al.*, 1989; Pestova *et al.*, 2001; Gebauer and Hentze 2004). In this process the rate-limiting step is the translation initiation, in which the loading of the small (40S) ribosomal subunit onto the mRNA is dependent upon the recruitment of multiple translation initiation factors (eIFs), including eIF4E, eIF4A and eIF4G, to form the translation initiation factor 4F (eIF4F) complex at the 5' cap structure of the mRNA (Ma and Blenis, 2009). In this context, the mTOR complex 1 (mTORC1) is part of the molecular machinery that couples growth factor stimulation to translation initiation by promoting the assembly of the eIF4F complex and enhancing the scanning activity of the small ribosomal subunit (Martin and Blenis, 2002). Therefore, we speculated that hScrib might modulate cap-dependent protein translation through the regulation of components of the mTORC1 pathway. To assess this possibility, we decided to investigate the activity of the PDK1 and S6 kinases, two protein kinases known to modulate the

mTORC1 pathway (Pullen and Thomas; 1997; Williams *et al.*, 2000). Figure 30a shows a cartoon of the mTORC1 pathway, and, as can be seen, the activity of PDK1 and S6 kinase is regulated by phosphorylation events. The autophosphorylation of PDK1 at serine 241 is crucial for its full activation, which is required upstream of mTOR for the activation of the pathway through the PI3K signaling (Casamayor *et al.*, 1999). On the other hand, mTOR directly activates its effector S6 kinase through the phosphorylation of its threonine 389; in turn, activated S6 kinase promotes protein synthesis by modulating the activity of multiple proteins involved in translation. HeLa cells were transfected with either siRNA Luciferase, or siRNA hScrib and after 72 hours the cells were harvested and levels of PDK1, S6 kinase and Akt were assessed by western blot by using antibodies to detect the total as well as phosphorylated forms of the different kinases. As can be seen in Figure 30b, HeLa cells express high levels of phospho-serine 241 (pS241) PDK1, and, surprisingly, the ablation of hScrib resulted in a reduction of both the phosphorylated and total PDK1 levels, suggesting that the reduced levels of phosphorylation are a reflection of the lower total protein levels. The activation of PDK1 results in the phosphorylation of Akt at threonine 308, which for its full activity also requires the mTORC2-mediated phosphorylation of serine 472 (Alessi *et al.*, 1996; Scheid *et al.*, 2002). The two phosphorylation events are sequential, however which of the two sites needs to be phosphorylated first to allow the subsequent full activation is still not completely clarified. Upon reduction of PDK1 expression due to the loss of hScrib, the levels of phosphorylated T308 as well as S473 Akt were also reduced, suggesting that the PDK1-mediated phosphorylation might play a crucial role in the full activation of Akt (Toker and Newton, 2000). Furthermore, this also indicates that the expression of hScrib might positively regulate the PI3K pathway. In the case of S6 kinase, this protein is expressed as two isoforms, p70 and p85, which share the same sequence with the exception that the p85 isoform has an extended N-terminus encoding a nuclear localization signal that promotes its nuclear accumulation (Pullen and Thomas, 1997). The mTORC1-mediated phosphorylation of a threonine residue which is located at position 389 in p70 and at position 412 in p85 is most critical for their function *in vivo* (Weng *et al.*, 1998); therefore we also monitored this phosphorylation event upon silencing of hScrib in HeLa cells. Similar to PDK1, the ablation of hScrib also led to the downregulation of total and phosphorylated

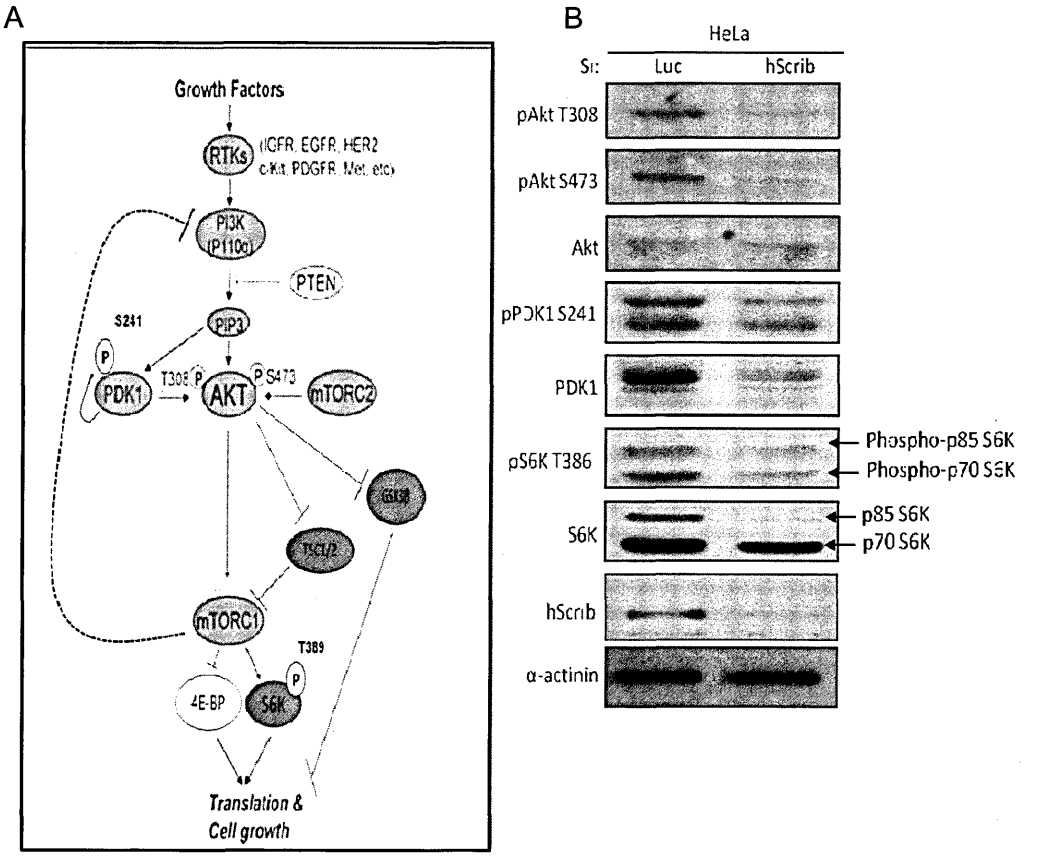

Figure 30. The expression of hScrib in HeLa cells maintains high levels of total PDK1 and S6 kinase levels. A. A cartoon summarizing the main components of the mTORC1 pathway is shown. The relative position of the components along the pathway as well as activating phosphorylation events are depicted. Adapted from Cheng and Force, 2010. B. HeLa cells were transfected with siRNA Luciferase or siRNA hScrib. 72 hours after transfection cells were harvested and the patterns of expression of total and phosphorylated levels of Akt, PDK1 and S6 kinase as well as those of hScrib and α-actinin as loading control, were assessed by western blot.

p70 and p85 S6 kinase. Previous studies indicated that the expression of HPV-16 E6 in human keratinocytes is sufficient to promote the activation of PDK1 and S6 kinase (Spangle and Mönger, 2010), therefore we decided to also include siRNA against HPV-18 E6 in the analysis, in order to rule out the possibility that effects on PDK1 and S6 kinase activity produced by the ablation of hScrib are indirectly linked to a reduction of E6 levels. Since previous studies suggested that E7 can also affect the levels of components of the PI3K pathway (Pim *et al.*, 2005; Menges *et al.*, 2006; Charette and McCance, 2007), we decided to use an siRNA that targets the intronic region of the E6 ORF. This strategy has been shown to efficiently inhibit the expression of HPV-18 E6 whilst only marginally affecting the expression of HPV-18 E7 and E6* (Butz *et al.*, 2003), which is consistent with the fact that type-I transcripts encoding the full-length E6 and E7 proteins are expressed at significantly lower levels than type-II and III transcripts, which encode E6* and E7 (Schneider-Gädicke and Schwarz, 1986). As can be seen in Figure 31a, silencing of HPV-18 E6 produced a reduction in levels of total and phosphorylated PDK1 comparable to that obtained by the ablation of hScrib. This indicates that the effect on PDK1 expression could be, at least in part, linked to the reduction in E6 levels upon loss of hScrib in HeLa cells. Interestingly, however, the silencing of E6 did not downregulate total and phosphorylated S6 kinase protein levels compared with siScrib cells, suggesting that hScrib might be regulating protein translation through the modulation of the S6 kinase activity.

The fact that loss of hScrib expression led to a reduction of total S6 kinase levels might indicate that this is linked to increased turnover of the protein. To assess whether the reduced levels of S6 kinase might result from increased degradation, we repeated the silencing of hScrib in HeLa cells in the presence of the proteasome inhibitor MG-132. 72 hours posttransfection, siLuciferase or siScrib HeLa cells were left untreated or treated with MG-132 for an additional 3 hours prior to harvesting. The levels of total and phosphorylated forms of PDK1, S6 kinase and also HPV-18 E6 were assessed by western blotting. The results are shown in Figure 31b, and, as can be seen, the ablation of hScrib again led to the efficient reduction in the levels of E6 expression. The proteasome inhibition led to a strong accumulation of HPV-18 E6 levels in siLuciferase HeLa cells,

which is in agreement with previous studies suggesting that E6 is regulated by the proteasome in a E6AP-dependent manner (Stewart *et al.*, 2004). Interestingly, upon silencing of hScrib the proteasome inhibition did not induce a rescue of E6 protein levels, which is consistent with the result in Figure 28 showing that loss of hScrib does not affect E6 protein stability. Upon proteasome inhibition total and phosphorylated PDK1 were upregulated in siLuciferase HeLa cells, suggesting that PDK1 is also regulated through the proteasome. In hScrib-silenced cells, however, PDK1 displayed a pattern of expression similar to E6, with the MG-132 treatment failing to rescue total and pS241 levels. Conversely, total S6 kinase levels were only marginally modified upon proteasome inhibition, both in siLuciferase and siScrib HeLa cells, indicating that its expression is most likely not regulated through the proteasome. However when the levels of phosphorylated S6 kinase were monitored upon MG-132 treatment, phospho-p85 S6 kinase levels were massively increased in both siLuciferase and siScrib transfected cells whereas phospho-p70 remained relatively unaffected. These data suggest that total and phospho-p70 are not regulated by the proteasome, whereas phospho- and total p85 display a differential mechanism of regulation and loss of hScrib might promote the proteasome-mediated degradation of phospho-p85 S6 kinase.

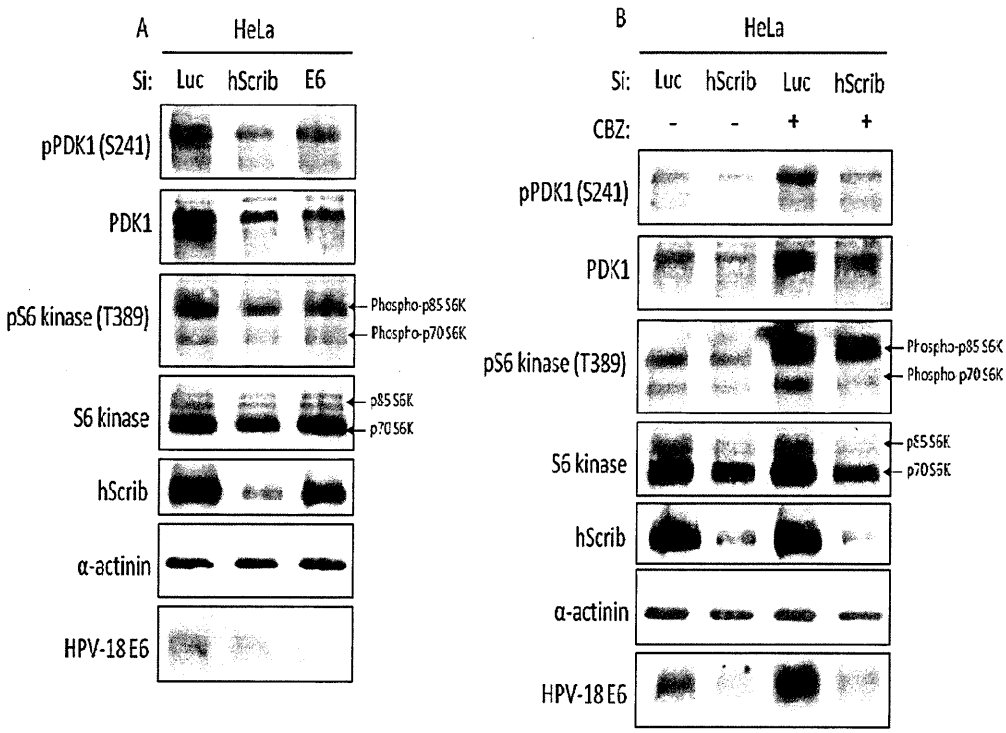

Figure 31. HPV-18 E6 regulates the expression levels of PDK1 but not those of S6 kinase. A. HeLa cells were transfected with siRNA Luciferase, siRNA hScrib and siRNA E6. 72 hours after transfection cells were harvested and the expression patterns of total and phosphorylated PDK1 and S6 kinase, as well as those of hScrib, HPV-18 E6 and α-actinin as loading control, were assessed by western blot. B. The siRNA transfection was performed as in A, but cells were treated with the proteasome inhibitor MG-132 for an additional 3 h prior to harvesting. The expression pattern of total and phosphorylated PDK1 and S6 kinase, as well as those of hScrib, HPV-18 E6 and α-actinin as loading control, were assessed by western blot.

Discussion

Part I:

E6-mediated regulation of PDZ domain-containing proteins in HPV-positive cells

A number of PDZ domain-containing substrates of E6 have been described, and many of these proteins are involved in diverse regulatory pathways, including the assembly of cell-cell junctions and cell attachment, and in the control of cell signaling. Potential tumor suppressor activities have also been assigned to several of these proteins. An important question that remains to be answered is whether or not all of these substrates are equally susceptible to E6-induced degradation *in vivo,* and how the relevant pathways they regulate might contribute to HPV-related malignancy. In an attempt to provide an answer to that, in our analysis we used specific siRNAs to silence the expression of E6 and E7 and E6AP in HPV-16 and HPV-18 transformed cell lines with the aim of identifying potential E6 PDZ domain-containing substrates relevant for HPV carcinogenesis *in vivo*. Recent structural studies had shown that the PDZ recognition by the HPV-16 and HPV-18 E6 PBMs requires very defined structural features, and minimal variations in the sequences of either the PBM or of the target PDZ domains has dramatic effects upon the pattern of substrate selection, as well as in the recognition of specific PDZ domains within the same protein (Zhang *et al.*, 2007). This is also supported by *in vitro* studies in which hDlg, hScrib and MAGI-1 were shown to be differentially selected for degradation by HPV-16 and HPV-18 E6 oncoproteins, with the former being more efficient for binding to hScrib, whereas HPV-18 E6 displays an increased affinity for hDlg and MAGI-1 (Gardiol *et al.*, 1999; Pim *et al.*, 2000; Thomas *et al.*, 2001; Thomas *et al.*, 2005). Although residues located upstream of the canonical PBM of E6 oncoproteins have been shown to contribute to the interaction with PDZ domains (Zhang *et al.*, 2007; Thomas *et al.*, 2008a), the major contribution towards the differential selection of these PDZ proteins is brought by the last residue of their PBM; respectively V and L in HPV-18 and -16 E6. This was further underlined by recent biacore measurements of the K_d values for the association of MAGI-1 PDZ domain 1 with the PBMs of HPV-16 and HPV-18 E6. These studies suggested that by mutating the HPV-16 E6 PBM into that of HPV-18 E6 (-ETQL→V) the affinity of MAGI-1 PDZ domain 1 for

HPV-16 E6 could be increased of three folds (Fournane *et al.*, 2011). Similarly, by swapping the two PBMs HPV-16 E6 becomes more efficient in degrading hDlg and MAGI-1, whereas HPV-18 E6 increases its efficiency in degrading hScrib (Thomas *et al.*, 2001; Thomas *et al.*, 2005). In agreement with all these studies, the data presented in Figures 10, 11 and 12 strongly suggest that the proteolytic degradation of PDZ proteins by E6 in HPV-transformed cells, is a highly specific process that involves the targeting of a specific subset of host-encoded PDZ domain-containing proteins.

In our study, we found that the levels of MAGI-1 expression are very low in HPV-16 and HPV-18-positive cells, and increased significantly following ablation of E6/E7 expression. As shown in Figure 11, the silencing of E6 and E7 led to a dramatic rescue of MAGI-1 protein levels in HeLa and SiHa cells, whereas the rescue in CaSKi was somewhat more marginal. However, the fact that MAGI-1 levels of expression were efficiently rescued in SiHa, but not in CaSKi, upon ablation of HPV-16 E6 and E7 suggests that the low levels of MAGI-1 expression in CaSKi cells might not be directly dependent upon HPV-16 E6 expression, and indicates that MAGI-1 is a sensitive proteolytic substrate for both HPV-18 and HPV-16 E6 oncoproteins *in vivo*. A marked difference in the pattern of HPV-16 and HPV-18 E6 PDZ substrate selection appears to be particularly relevant for some other potential targets, including hDlg, hScrib and PSD95. An interesting aspect of hScrib and hDlg expression in HPV-positive cells is that their levels are readily detectable in HeLa and CaSKi cells. Upon ablation of E6 and E7, hScrib appears to be rescued more efficiently in CaSKi than in HeLa cells (Figure 10a and 10c), whereas hDlg showed the higher degree of rescue in HeLa cells at 48 and 72 hours post transfection (Figure 10a and 10b). However, an increase in hDlg levels became more apparent at 72h after transfection also in CaSKi cells (Figure 10b), suggesting that HPV-16 E6 retains the ability to promote the degradation of hDlg in HPV-transformed cells. It is also interesting to note that upon silencing of E6 and E7 the pattern of hDlg expression differed between HeLa and CaSKi cells, likely representing differentially modified forms or different isoforms of hDlg. This suggests that both HPV-16 and HPV-18 E6 target hDlg for degradation *in vivo*, but they might differ in the selection of the pools of hDlg targeted for

degradation. Similarly, we found that the hDlg-related protein, PSD95, is efficiently targeted by HPV-18 E6, but less so by HPV-16 E6 (Figure 12b), and this is in agreement with previous reports (Handa *et al.*, 2007). Silencing of E6AP largely confirmed the differential PDZ substrate selection pattern between HPV-16 and HPV-18 E6. It would be of interest to better define the contribution of E6AP towards the degradation of E6 PDZ substrates, since previous studies reported that E6 can degrade its substrates also in a E6AP-independent manner (Massimi *et al.*, 2008b). However, in our experimental setting it is hard to obtain conclusive evidence about the involvement of E6AP in the E6-mediated degradation of its substrates, since loss of E6AP expression has been shown to greatly affect E6 stability (Tomaić *et al.*, 2009), making it virtually impossible to differentiate their respective contribution towards the degradation of E6 substrates under these experimental conditions.

Of the remaining PDZ substrates of E6 that were analysed, we failed to obtain conclusive evidence that PTPN3 (Figure 12a) or TIP2 (Figure 12c) were targeted for degradation either by HPV-16 or HPV-18 E6 in monolayer cultures of cells derived from cervical tumors and. This is in marked contrast with previous studies suggesting that TIP2 and PTPN3 are targeted for proteasome-mediated degradation by high-risk HPV E6 oncoproteins (Favre-Bonvin *et al.*, 2005; Töpffer *et al.*, 2007; Jing *et al.*, 2007). However, these studies do not rule out the possibility that these PDZ domain-containing proteins may be degradation substrates of E6 in other biological settings: during different stages of the normal viral life cycle where the cells are subject to terminal differentiation, or at an earlier stage of tumour development. This might reflect differences in the phosphorylation status of the target protein, which could influence accessibility to E6 and subsequent targeting (Massimi *et al.*, 2006; Narayan *et al.*, 2009). Finally, we should also emphasize that, although degradation has been proposed as a major mechanism by which E6 exerts its function, it is possible that some of these substrates may be only bound by E6, and that blocking a certain PDZ substrate-ligand interaction might be sufficient for E6 to modulate the function of that particular cellular PDZ domain-containing protein, or alternatively, E6 might also alter the localization of the substrate.

The last E6 PDZ domain-containing substrate analysed in our study was the non-receptor tyrosine phosphatase PTPN13 or FAP-1. We included this protein in our analysis since previous studies described FAP-1 as a proteolytic target for the HPV-16 E6 oncoprotein in tonsillar keratinocytes (Spanos *et al.*, 2008b). In our analysis we detected very low levels of FAP-1 expression in HeLa cells, whereas higher FAP-1 protein levels were expressed in CaSKi cells. Surprisingly, the expression of FAP-1 was downregulated in both HPV-positive cell lines upon ablation of E6 and E7, whereas its levels were unchanged upon silencing of E6AP in CaSKi cells (Figure 12d). This pattern of expression in CaSKi cells would fit with a possible involvement of E7 in the regulation of FAP-1. This possibility is particularly intriguing since two sequences matching the putative E2F-binding site TTTSSCGC (where S is C or G) are present in the promoter region of FAP-1 (C.K. personal observation; Abaan and Toretsky, 2008). This suggests that that HPV-16 E7 could indirectly drive the expression of FAP-1 through the destabilization of E2F/pRB complexes.

Considering the differences in the regulation of FAP-1 by HPV E6 in head and neck and cervical cancer-derived cells, it is important to note that there appear to be some significant differences between HPV-mediated head and neck and cervical carcinogenesis. Recent studies in which HPV-16 E6 and E7 were expressed in head and neck tissues, under the control of the K14 promoter, suggested that in head and neck tumors the carcinogenic potential of HPV-16 is less dependent upon the ability of E6 to interact with E6AP and its PDZ domain-containing substrates (Jabbar *et al.*, 2010) compared with models for cervical carcinogenesis (Shai *et al.*, 2007a ; Shai *et al.*, 2010), indicating that in different tissues HPV oncoproteins might follow different routes of oncogenic transformation. In addition, these differences are also underlined by the fact that although the p16 expression is generally accepted as a surrogate marker for cervical cancer progression (Tsoumpou *et al.*, 2009), the correlation between the p16 overexpression and HPV-positive cancers in different head and neck tissues appears not to be unequivocal (Hoffaman *et al.*, 2012), possibly reflecting tissue specific differences.

FAP-1 function appears to be highly pleiotropic and context-dependent. Consistent with this, several reports ascribe to FAP-1 both tumor suppressive and tumor promoting functions (Abaan

and Toretsky, 2008). A relevant aspect of FAP-1 biological activity is its effect on Fas-mediated apoptosis. The activation of the Fas (CD95/APO-1) receptor, which is exposed on the cell surface, occurs through the engagement of the Fas ligand (FasL/CD95L), a trimeric protein belonging to the tumor necrosis factor (TNF) superfamily, ultimately resulting in the induction of caspase-8-medited apoptosis. Expression of FAP-1 has been correlated either with a decreased expression of FasR on the cell surface (Ungefroren *et al.*, 2001; Ivanov *et al.*, 2001) or with the deregulation of the Fas-associated pro-apoptotic signaling (Meinhold-Heerlein *et al.*, 2001; Foehr *et al.*, 2005; Wieckowski *et al.*, 2007), thereby prolonging the survival of FAP-1 expressing cells. The maintenance of FAP-1 expression in the context of viral life cycle could then represent an additional strategy to escape apoptosis or evade the immune system surveillance, since the Fas-associated signaling is one of the prime mediators of T-cell-mediated cytotoxicity towards virus-infected cells (Ju *et al.*, 1994). This hypothesis is also reinforced by the fact that HPV-16 E5 has been shown to downregulate FasR protein levels and its membrane targeting. In addition, other viruses, including adenovirus, KSHV and HTLV-1, have evolved similar mechanisms to block Fas-mediated apoptosis (Shisler *et al.*, 1997; Tollefson *et al.*, 1998; Belanger *et al.*, 2001; Okamoto *et al.*, 2006). Obviously, further studies are required to determine whether E7 can upregulate FAP-1 in an E2F-dependent manner.

Since biochemical data suggest that MAGI-1 is the strongest interacting partner of HPV E6 (Thomas *et al.*, 2001, Zhang *et al.*, 2007) we focused on further defining the relevance of MAGI-1 degradation in HPV-induced malignancy. MAGI-1 is a TJ-associated protein whose best understood function is its ability to promote TJ assembly (Hirabayashi *et al.*, 2003; Murata *et al.*, 2005). However, the sequence analysis of MAGI-1 reveals the presence of a strong bipartite nuclear localization signal in the carboxy terminus of the protein (Dobrosotskaya *et al.*, 1997), consistent with there being a pool of MAGI-1 normally resident within the nucleus of epithelial cells (Dobrosotskaya and James, 2000; Kranjec and Banks, 2011). Thus, this complex pattern of distribution suggests that MAGI-1 is likely to be involved in additional biological processes beside its ability to promote the assembly of junctional complexes. Using differential cell fractionation we verified that the abolition of E6/E7 expression restores MAGI-1 expression at two main locations within HPV-positive cells: the cell membrane and the cell nucleus (Figure 13a). This suggests that

whatever functions these two pools of MAGI-1 perform, the removal of one or both has advantages for the virus. Fractionation experiments in HPV-negative cells, however, revealed high levels of MAGI-1 expression also in the cytoplasm (Figure 13b), suggesting that the pattern of MAGI-1 localization is likely to be dynamic and cell-type specific. There are currently no studies available that could offer an explanation for what MAGI-1's function is in the nucleus. Further studies will aim at more fully defining the functions of this form of the protein.

In contrast, the membrane-bound form of MAGI-1 has been implicated in the control of TJs (Murata *et al.*, 2005), which are lost in HPV-positive cells (Nakagawa and Huibregtse, 2000; Latorre *et al.*, 2005; Storrs and Silverstein, 2007). The results reported here show that this loss is indeed a result of E6 directing the degradation of MAGI-1. Using ZO-1 as a marker of TJ integrity, we have confirmed that these junctions are largely absent in HPV-18-positive HeLa cells, and that ablation of E6 expression results in a clear re-accumulation of MAGI-1 at the cell membrane, accompanied by an accumulation of ZO-1 at the same cellular location (Figure 14). Interestingly, this appears to be a slow process, with a bead-like structure, indicative of the early stages of junction formation (Kimura *et al.*, 2010), apparent at 72h after transfection with E6/E7 siRNA, and more complete junctions visible by 96h. To verify that restoration of TJs depends upon the rescue of MAGI-1 from E6-induced degradation, we co-transfected a MAGI-1 siRNA with the E6/E7 siRNA, and in this case there was no evidence of TJ formation (Figure 14). Recent studies suggested that hScrib is also strongly implicated in the process of epithelial TJ assembly (Nakagawa and Huibregtse, 2000; Ivanov *et al.*, 2010; Elsum *et al.*, 2013). However, upon silencing of hScrib in combination with E6 and E7 in HeLa cells we failed to detect any deleterious effect upon ZO-1 junctional recruitment (Figure 15a), confirming the specificity of the results with MAGI-1.

Potential roles played by MAGI-1 in HPV-related disease

A particularly interesting structural insight in the context of MAGI-1 regulation by E6, has been the identification of lysine 499 within the PDZ-1 domain as a crucial residue required for the interaction with the PBM of HPV-16 and HPV-18 E6 oncoproteins (Fournane *et al.*, 2011).

Consistent with this, mutating K499 to E in the context of full length MAGI-1, we were able to dramatically decrease the affinity of MAGI-1 for HPV-18 and HPV-16 E6 oncoproteins (Figure 16c and 16d), and this also correlated with a reduced ability of E6 to induce the proteasome-mediated degradation of MAGI-1, *in vitro* and *in vivo* (Figure 17). The PDZ domain 1 of MAGI-1, has also been identified as the interacting region for the PBM of multiple cellular proteins, and one of such binding partner is NET1 (Dobrosotskaya, 2001). NET1 is a RhoA-specific guanine nucleotide exchange factor which is involved in a number of cancer-associated biological processes, including cell migration, proliferation and matrix invasion (Murray *et al.*, 2008; Han *et al.*, 2012). By performing co-immunoprecipitation assays we confirmed that NET1 is a binding partner for wild type MAGI-1 *in vivo*, and that the K499E mutation also strongly affects the interaction of MAGI-1 with NET1 (Figure 18a). However, the K499E mutation might not completely disrupt the functionality of the PDZ 1 domain, as the interaction with HPV-58 E6 was shown to be not affected by the mutation (Figure 18c). Furthermore, by performing immunofluorescence on wild type or K499E mutant MAGI-1-transfected cells, we showed that the mutation does not significantly modify the pattern of subcellular localization of MAGI-1, and also does not affect its interaction with a membrane-bound protein such as β-catenin (Figure 19).

The generation of the K499E MAGI-1 mutant provided a valuable molecular tool for exploring its role in the context of HPV-related pathology, since it allowed us to evaluate the effects of reintroducing MAGI-1 expression in HPV-positive cells without the need to silence E6 and E7 expression. By transiently expressing wild type and mutant MAGI-1 in HeLa cells, we confirmed that MAGI-1 expression alone is sufficient to drive TJ reassembly in HPV-positive cells, which was assessed by monitoring the expression of ZO-1 and another TJ marker, PAR3 (Figure 20a, 20b and Figure 21). However, both ZO-1 and PAR3 have a dynamic pattern of junctional localization, and have been shown to co-localize with AJ components during the induction of primordial AJ structures following initial cell-cell contact (Ando-Akatsuka *et al.*, 1999; Suzuki *et al.*, 2002). Therefore, we cannot rule out the possibility that the ZO-1- and PAR3-positive junctional structures observed in MAGI-1-expressing cells are infact primordial AJs. Nevertheless, these data strongly suggest that MAGI-1 directly participates in the establishment of macromolecular

complexes that localize at the TJ plaque in fully polarized cells. PAR3 is part of the PAR complex which localizes and controls the maturation of TJs, however PAR3 has been shown to be able to promote TJ assembly independently of PAR6 and aPKC (Chen and Macara, 2005), suggesting that PAR3 is one of the prime regulators of TJ formation. Therefore, the fact that MAGI-1 enhances PAR3 junctional localization strongly suggests the involvement of MAGI-1 in the establishment of cell polarity by inducing the formation of apical junctional structures (Murata *et al.*, 2005). In support of this, the expression of MAGI-1 and PAR3 have been shown to have similar effects on the recruitment of occludin at TJs (Hirabayashi *et al.*, 2003; Chen and Macara, 2005). However, so far a direct interaction of MAGI-1 with either ZO-1 or PAR3 has not been shown, suggesting that their MAGI-1-mediated junctional recruitment is likely to occur in an indirect way. MAGI-1 as well as ZO-1 and PAR3 have all been shown to interact with JAMs which are enriched at the TJs of epithelial cells (Ebnet *et al.*, 2000; Bazzoni *et al.*, 2000; Itoh *et al.*, 2001; Ebnet *et al.*, 2003; Hirabayashi *et al.*, 2003). The interaction of the cytoplasmic domain of JAM molecules with components of the TJ plaque has been shown to promote their stabilization. Therefore it is likely that the expression of MAGI-1 might promote the initial clustering of JAM molecules at the cell membrane (Hirabayashi *et al.*, 2003), which subsequently also drives the recruitment of ZO-1 and PAR3. Figure 32 summarizes the role of MAGI-1 in maintaining TJ integrity as well as in regulating proliferation and apoptosis, and describes the effects of E6 expression in the context of MAGI-1 activity.

These results demonstrate that the loss of TJs in HPV-18 positive HeLa cells is a direct consequence of the ability of E6 to direct the degradation of MAGI-1, and might provide a an explanation of why this protein is targeted by the virus during the life cycle and by E6 in malignancy.

TJs play an important role in differentiation where their correct assembly promotes the exit from the cell cycle and contributes to keratinocyte differentiation (Saitou *et al.*, 2000; Bordin *et al.*, 2004; Aijaz *et al.*, 2005). Loss of TJs can therefore be expected to delay the differentiation process. In addition, TJs directly participate in the regulation of cell proliferation by modulating signaling

cascades such as MAPK, PKB/Akt and RhoA signaling (Li and Mrsny, 2000; Kotelevets *et al.*, 2005; Aijaz *et al.*, 2005). Interestingly, both ZO-1 and PAR3 are believed to be involved in controlling cell proliferation; while ZO-1 binds and sequesters the transcription factor ZONAB/DbpA at the cell membrane (Balda *et al.*, 2003), PAR3 is believed to potentially regulate cell proliferation through the modulation of the p53-binding partner ASPP2 (Sottocornola *et al.*, 2010). In our cell proliferation assay, we found that the expression of wild type MAGI-1 had dramatic effects upon the proliferative potential of HeLa cells (Figure 22). However this effect was shown to be largely independent of the junctional recruitment of ZO-1 and PAR3. Consistent with this, the K499E mutant MAGI-1 was less efficient than the wild type protein in inhibiting cell proliferation yet displaying the same efficiency as the wild type protein in promoting ZO-1 and PAR3 junctional recruitment. Most importantly, the effects of MAGI-1 upon cell proliferation appear to be specific for HPV-positive cells, since the transfection of wild type and mutant MAGI-1 into HaCaT cells had a much weaker effect upon cell proliferation (Figure 23).

This suggests that MAGI-1 can regulate cell proliferation through a mechanism not involving its junctional recruitment. An intriguing possibility is that MAGI-1 might regulate cell proliferation, at least in part, through the modulation of RhoA activity (Figure 32a). This is supported by the fact that MAGI-1 interacts with the RhoA-specific activator NET1, and that MAGI-1 had already been shown to modulate the activity of another small GTPase Rap1, with consequent stabilization of vascular-endothelial cell adhesion structures (Mino *et al.*, 2000; Sakurai *et al.*, 2006;). In addition, RhoA and NET1 have already been described as positive regulators of cell cycle progression (Leyden *et al.*, 2006; Han *et al.*, 2012). The decreased ability of the K499E mutant MAGI-1 to inhibit cell proliferation would then be consistent with its reduced capacity to interact with NET1. It is also interesting to note that recent studies had shown that NET1 is mainly localized in the nucleus of epithelial cells, where its activity is required to maintain RhoA in an active GTP-bound state (García-Mata *et al.*, 2007; Dubash *et al.*, 2011). Although the effect that the MAGI-1 could have on NET1 is still not clear, these data potentially provide a possible biological function for the nuclear pools of MAGI-1 in regulating RhoA activity, and might suggest that the targeting of

nuclear pools of MAGI-1 contributes to increase the proliferation of HPV-positive cells (Figure 32b).

Our data also suggest a new function for MAGI-1 in the induction of apoptosis of HPV-positive cells. We hypothesized a possible involvement of MAGI-1 in the regulation of apoptosis since upon expression of wild type and K499E mutant MAGI-1 in HeLa cells, we noticed that a proportion of MAGI-1-positive cells were also displaying a morphology typically associated with apoptosis. In order to determine whether MAGI-1 could indeed promote apoptosis we performed TUNEL assays on HeLa cells transiently transfected either with wild type or K499E MAGI-1 constructs (Figure 24a). Our data strongly suggest that both the wild type and mutant MAGI-1 can promote the apoptosis of HeLa cells. It is also interesting to note that the K499E mutant MAGI-1 displayed an efficiency in the induction of apoptosis that was comparable to that of the wild type protein. Therefore, this might suggest that the regulation of apoptosis by MAGI-1 is not dependent upon functions associated with the PDZ1 domain. Furthermore, as for the induction of junctional assembly in HeLa cells, the increased resistance of the K499E mutant MAGI-1 to E6-mediated degradation also correlated with an increased subpopulation of HeLa cells undergoing apoptosis (Figure 24a and Figure 25).

Therefore, taken together these data strongly indicate that the E6-mediated degradation of MAGI-1 could represent an additional mechanism evolved by high-risk HPV types to escape apoptosis.

Previous studies had suggested that TJ assembly might also be implicated in the regulation of apoptosis. It is interesting to note that the PAR3 binding partner ASPP2 has been shown to be strongly implicated in the induction of apoptosis by promoting the p53-mediated transactivation of pro-apoptotic p53 target genes, including *Bax*, *PUMA*, and *Fas/CD95* (Samuels-Lev *et al.*, 2001; Wilson *et al.*, 2013). In addition, given the fact that ASPP2 localizes at cellular junctions in a PAR3-dependent manner (Sottocornola *et al.*, 2010), it is tempting to speculate that the MAGI-1-induced junctional recruitment of PAR3 could play some role in the pro-apoptotic activity of ASPP2. It is also interesting to note that the inactivation of p53 by E1B did not affect the ability of ASPP2 to induce apoptosis (Kobayashi *et al.*, 2005), suggesting that ASPP2 can induce apoptosis

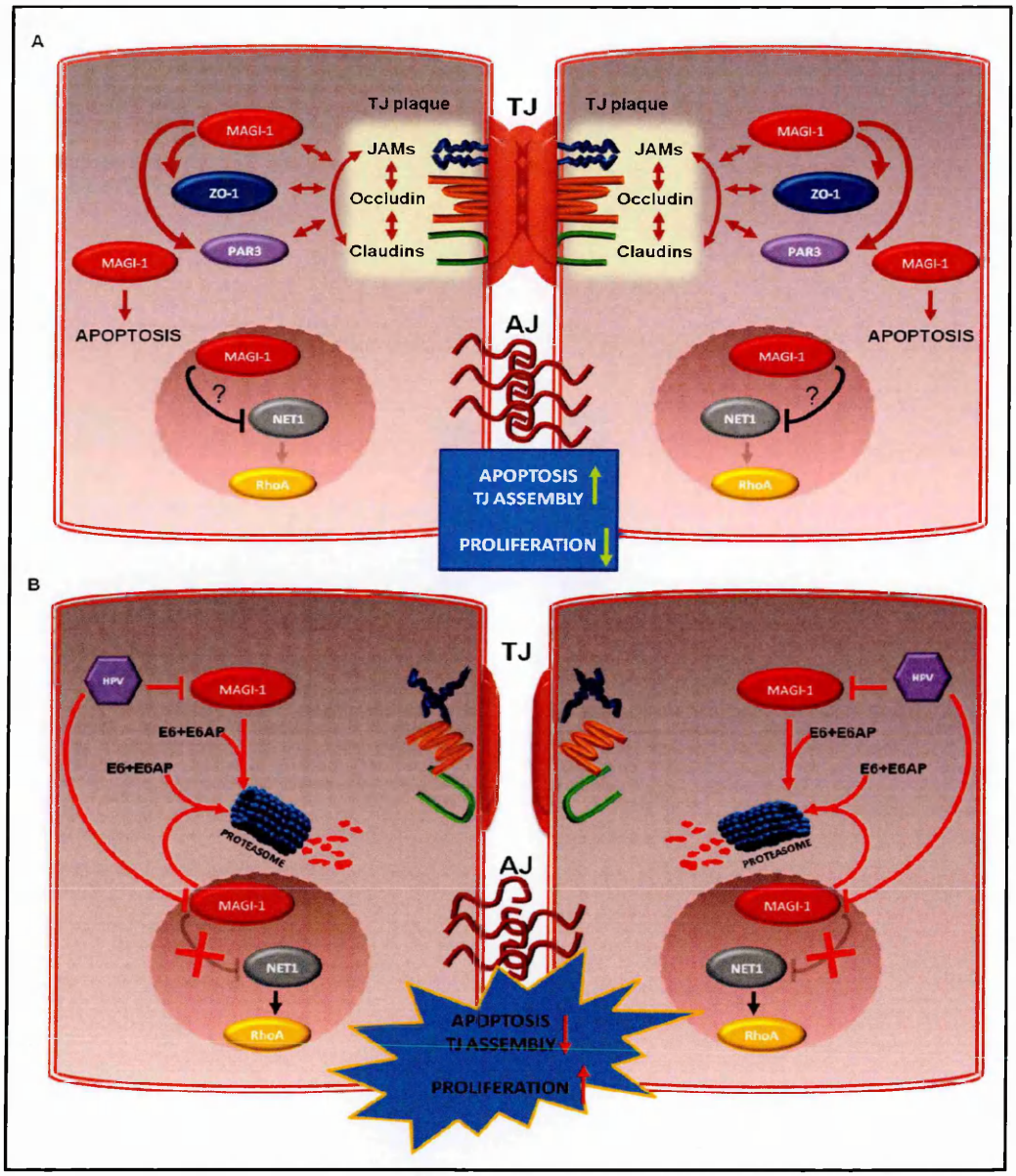

Figure 32. A. Cartoon summarizing the regulation of TJ integrity and apoptosis by MAGI-1. A possible role for the nuclear pools of the protein in inhibiting RhoA activity is also depicted. Double arrows refers to the interdependent regulation of TJ proteins. B. Infection with high risk HPV and expression of E6 leads to the proteasome-mediated degradation of membrane-bound and nuclear pools of MAGI-1, resulting in the destabilization of junctional complexes and loss of junctional integrity. This might also potentially lead to the loss of MAGI-1-medated NET1 regulation with downstream effects on RhoA activity.

in a p53-independent manner. In addition, recent studies showed that loss of occludin mediated the resistance of squamous cell carcinoma cells to apoptotic stimuli (Rachow *et al.*, 2013), suggesting that the maintenance of TJ integrity can play important roles in the regulation of apoptosis.

Part II:

Regulation of HPV-18 E6 expression by hScrib

From the data presented in this thesis and elsewhere (Thomas *et al.*, 2001; Nguyen *et al.*, 2003a; Thomas *et al.*, 2005; Simonson *et al.*, 2005; Shai *et al.*, 2007; Kranjec and Banks, 2011) it is clear that the targeting of PDZ domain-containing proteins by E6 plays an important role in the pathogenesis of cervical cancer. In addition, E6 oncoproteins can select specific PDZ proteins to target for proteasome-mediated degradation and recent studies indicated that different subcellular pools of PDZ proteins are subjected to differential regulation by E6 (Narayan *et al.*, 2009; Krishna Subbaiah *et al.*, 2012). Increasing evidence also suggests that certain PDZ domain-containing proteins or specific subcellular pools can cooperate with E6 in the maintenance of the transformed phenotype (Krishna Subbaiah *et al.*, 2012). In our study we identified an unexpected role for hScrib in the context of the HPV-related pathology through the regulation of HPV-18 E6 protein translation. Previous studies already suggested that the maintenance of high-risk HPV episomes in human keratinocytes is dependent upon the ability of E6 to interact with its PDZ domain-containing substrates (Lee and Laimins, 2004; Nicolaides *et al.*, 2011; Delury *et al.*, 2013). In addition, the interaction of HPV-16 E6 with multiple host encoded PDZ domain-containing proteins was shown to regulate its levels of expression, suggesting that this effect is mediated by the engagement of the E6 PBM with multiple PDZ proteins rather than involving a specific PDZ domain-containing substrate of E6. However, all of these assays involved overexpression settings, and an analysis of the potential regulation of E6 by PDZ domain-containing proteins at an endogenous level was still missing. By ablating a pool of PDZ domain-containing proteins in HeLa cells we found that only loss of hScrib expression reduced endogenous levels of HPV-18 E6 expression (Figure 26). This suggests that in the context of endogenously expressed protein only hScrib appears to modulate E6 levels of expression.

Before discussing the hScrib data in more detail, it is worth considering the results obtained in Figure 26 with the ablation of the other PDZ domain-containing proteins. Upon silencing of hScrib, hDlg, TIP2, PSD95 and PTPN3, we observed a general increase in E6AP expression levels. In the case of hScrib, the upregulation of E6AP expression could be related to the reduction of E6 expression, since previous studies showed that E6 can promote the proteasome-mediated degradation of E6AP (Kao *et al.*, 2000). However, the fact that the loss of the other PDZ proteins elevated E6AP levels, without affecting the expression of E6, could suggest a more direct involvement of these proteins in the regulation of E6AP levels. Similarly, p53 levels were shown to be increased upon the ablation of all of the PDZ proteins included in our analysis. So far, previous studies identified only TIP1 as a putative PDZ protein able to modulate p53 protein levels (Han *et al.*, 2012). Nevertheless, our data strongly suggest that the expression of additional PDZ proteins could modulate the pattern of p53 expression in HPV-positive cells, at least in part, in a E6-independent manner. Whether this is a general stress response triggered by the loss of these PDZ proteins, or a more specific effect remains to be determined. Furthermore, we also observed that there is a significant interplay between the different PDZ domain-containing proteins in HPV-positive cells. This is particularly evident in the case of hScrib, whose levels of expression were dramatically increased upon ablation of hDlg in HeLa cells. Although previous studies failed to observe a change in the levels of hScrib expression upon loss of hDlg (Ivanov *et al.*, 2010), our data suggest that compensatory effects between different PDZ domain-containing proteins might exist in HeLa cells.

Further analysis of the mechanisms through which hScrib exerts these effects on E6 expression appears to rule out an effect on E6 protein turnover, since its half-life was unchanged upon ablation of hScrib (Figure 28). Furthermore, in our fractionation experiments we failed to detect any variation in the pattern of subcellular localization of HPV-18 E6 upon loss of hScrib (Figure 27), suggesting that the pattern of E6 expression was not affected by loss of hScrib. This assay did however highlight a number of points about E6. First we confirmed that HPV-18 E6 displays a differential pattern of subcellular localizations, with the bulk of it being expressed at membrane bound sites (Grossman *et al.*, 1989). In addition, previous studies had shown that HPV-18 E6

promotes the nuclear export of p53 in order to drive its degradation primarily through the cytosolic proteasome pathway, although E6 can drive the nuclear degradation of p53 albeit with lower efficiencies (Freedman and Levine, 1998; Stewart *et al.*, 2005). We found that p53 localizes in nuclear as well as membrane fractions in siLuciferase transfected HeLa cells, and the reduction of the levels of E6 expression induced by the loss of hScrib expression led to an increase in p53 protein levels in both nuclear and in extra-nuclear fractions of HeLa cells, confirming that HPV-18 E6 can degrade different pools of p53. However, this does not appear to fit with the E6-mediated relocalization of p53. We also observed that HeLa cells express high levels of nuclear hScrib and E-cadherin. Previous studies have defined that the tumor-suppressive functions of hScrib are highly dependent upon its membrane localization, and consistent with this the displacement of hScrib from the membrane to the cytoplasm has been described to interfere with hScrib pro-apoptotic activity (Liu *et al.*, 2010; Zhan *et al.*, 2008) and with its ability to regulate the Hippo pathway (Cordenonsi *et al.*, 2011). In HeLa cells we detected hScrib predominantly in the nucleus and in the membrane, with lower levels of expression present in the cytoplasm. Importantly, so far no nuclear localization for hScrib has been described, and it is interesting to speculate that E6 might play a role in the nuclear accumulation of hScrib, although the possible function of nuclear pools of hScrib remains to be determined. On the other hand, the aberrant cleavage of E-cadherin and the nuclear localization of its cytoplasmic domain have been correlated with the loss of the epithelial phenotype and acquisition of tumorigenic properties in epithelial cells (Ferber *et al.*, 2008; Chetty *et al.*, 2008; Salahshor *et al.*, 2008). However, recent studies detected high levels of full length E-cadherin expression in the nucleus of a proportion of metastatic colorectal cancer cells, indicating that the mislocalization of full length E-cadherin is potentially an important event during invasive cancer progression (Salahshor *et al.*, 2008). The nuclear expression of E-cadherin in HeLa cells might then be consistent with their high tumorigenic potential. The HPV oncoproteins are known to downregulate the E-cadherin expression by inducing hyper-methylation of its promoter (Laurson *et al.*, 2010; D'Costa *et al.*, 2012), however these data suggest that part of the residual E-cadherin expressed in HeLa cells might be aberrantly localized in the nucleus, possibly contributing to their tumorigenic potential.

Previous studies have shown that the pattern of endogenous HPV-18 E6 expression in HeLa cells can be modulated by some of its cellular binding partners, including E6AP and 14-3-3 proteins (Tomaić *et al.*, 2009; Boon and Banks, 2013), with loss of E6AP associated with a strong reduction of E6 half-life (Tomaić *et al.*, 2009). Ablation of hScrib in HeLa cells did not significantly affect E6 turnover or the transcription of its mRNA (Figure 28), but dramatically perturbed the recovery of HPV-18 E6 protein levels upon release from the inhibition of protein translation (Figure 29); this is consistent with the involvement of hScrib in the regulation of E6 translation, previously shown to be cap-dependent (Tan *et al.*, 1994; Stacey *et al.*, 2000). It is also interesting to note that although E6 and E7 are transcribed from the same mRNA, they appear to be translated through different mechanisms; with E7 translation displaying a reduced cap-dependency and being potentially translated through an IRES-dependent mechanism (Stacey *et al.*, 1995; Stacey *et al.*, 2000).

Intriguingly, we found that upon loss of hScrib the half-life of p53 (Figure 28a) was also significantly increased in HeLa cells, consistent with reduced levels of E6 expression, whereas its translation efficiency was largely unaffected (Figure 29a). However, p53 has been reported to be translated through both cap-dependent and cap-independent mechanisms due to the presence of a internal ribosome entry site (IRES) in its 5'-UTR (Ray *et al.*, 2006). Therefore, this suggests that the pattern of p53 expression upon loss of hScrib is linked to the reduced expression of HPV-18 E6 but is independent from the hScrib-regulation of protein translation.

As an attempt to define the molecular mechanism by which loss of hScrib could affect protein translation we analysed the effects on the phosphatidylinsositol-3 kinase (PI3K)/mammalian target of rapamycin complex 1 (mTORC1) pathway. This pathway is known to integrate the availability of nutrients present in the environment surrounding the cells to with the activation of anabolic pathways, and the stimulation of protein translation is the best understood mechanism through which the PI3K/mTORC1 pathway promotes cell growth and proliferation (Fingar and Blenis, 2004). Since the functionality of both pathways is required for the effective activation of protein translation, it is important to note that our study highlighted that E6 and hScrib might act synergistically to activate protein translation through the regulation of separate components of

PI3K and mTORC1 pathways. Our data suggest that silencing of E6 in HeLa cells leads to a reduction in the levels of PDK1 expression (Figure 31a). PDK1 is a direct downstream effector of PI3K, and one of the better understood functions associated with its activity is the phosphorylation of Akt in its activation loop (or T-loop) at threonine 308 (T308). Previous studies suggested that, upon activation of the PI3K pathway, the kinetics of Akt activation involve its mTORC2-mediated phosphorylation at S473, which in turn facilitates the subsequent T308 phosphorylation by PDK1 (Scheid *et al.*, 2002; Sarbassov *et al.*, 2005). Although alternative mechanisms of Akt activation have also been proposed (Toker and Newton, 2000), it is clear that the phosphorylation of Akt at both sites is a crucial requirement for its full activation (Alessi *et al.*, 1996; Scheid *et al.*, 2002). Previous studies suggested that the expression of HPV-16 E6 was sufficient to maintain high levels of active Akt (Spangle and Münger, 2010; Spangle and Münger, 2013), and this indicates that the E6-mediated upregulation of PDK1 expression might be in part responsible for driving high levels of Akt activity. It is also interesting to note that some of the biological activities associated with Akt function can be specifically linked to one of the activating phosphorylation events. For instance, while the S473 phosphorylation is required for Akt to regulate cell survival pathways, the PDK1-mediated T308 phosphorylation of Akt was suggested to be most critical for the activation of components of the mTORC1 pathway involved in protein translation (Jacinto *et al.*, 2006). Thus, our data suggest that the regulation of PDK1 levels by E6 might represent a prime mechanism through which E6 stimulates the PI3K/mTORC1 pathway and protein translation.

In addition, the expression of HPV-16 E7 has also been shown to drive the activation of Akt in organotypic raft cultures, and this activity was linked to the ability of E7 to inactivate pRB (Menges *et al.*, 2006). Moreover the E7-mediated activation of Akt was shown to induce cell migration in human keratinocytes by driving the Akt-mediated cytoplasmic mislocalization of p27 (Charette and McCance, 2007). This indicates that HPV oncoproteins coordinately induce a stimulatory effect on Akt, suggesting that they regulate a wide variety of biological processes through the modulation of PI3K pathway.

Previous studies had indicated that hScrib is a negative regulator of PI3K signaling. hScrib was shown to facilitate downregulation of Akt by promoting the membrane recruitment of the Ser/Thr protein phosphatase PHLPP1 (Li *et al.*, 2011). Although we failed to detect an upregulation of phosphorylated Akt upon loss of hScrib in HeLa cells (Figure 30b), we believe that decreased levels of phosphorylated Akt might be a reflection of decreased levels of PDK1 expression driven by the reduced levels of HPV-18 E6 in cells transfected with siRNA against hScrib. We hypothesize that hScrib might have a more direct effect on the pattern of total and phosphorylated p70 and p85 S6 kinase expression (Figure 31). The function of the p70 isoform of S6 kinase has been shown to be strongly implicated in the induction of cap-dependent protein translation and cell cycle progression, and its mTORC1-mediated phosphorylation at threonine 389 (T389) has been shown to be critical for these activities (Lane *et al.*, 1993; Jefferies *et al.*, 1997; Zhou *et al.*, 2011). Similarly, p85 S6 kinase can be phosphorylated by mTORC1 at the same position, however the biological consequences of the activation of p85 are less clear, although this isoform has also been linked to the induction of cell cycle progression (Reinhard *et al.*, 1994). The fact that hScrib seems to regulate the total levels of both isoforms of the S6 kinase might argue that hScrib directly regulates the levels of S6 kinase expression, rather than its activation downstream of activated mTORC1.

Taken together these data provide an unexpected function for hScrib in HPV-positive cells, and suggest that the maintenance of a critical level of hScrib expression in HPV-positive cell monolayers could, in part, contributes to the pro-oncogenic activity of E6 and E7 through the positive regulation of p70 and p85 S6 kinase, and this is also likely to contribute indirectly to hScrib-mediated regulation of translation, including that of HPV-18 E6.

It is also interesting to note that the treatment of HeLa cells with the proteasome inhibitor MG-132 produced a dramatic increase in the levels of phosphorylated p85, but not of p70, S6 kinase expression (Figure 31b). This suggests that the two isoforms of the S6 kinase are regulated through different mechanisms, and that the mTORC1-mediated phosphorylation of p85 enhances its proteasome-mediated degradation. In addition, upon ablation of hScrib, the proteasome inhibition

appears to rescue, at least in part, the levels of phosphorylated p85 S6 kinase expression, indicating that loss of hScrib might contribute to the proteolytic degradation of phosphorylated p85. Recent studies defined that p85 S6 kinase positively regulates apoptosis under oxidative stress, promoting the inhibition of mdm2 and accumulation of p53 (Jia *et al.*, 2013). Although this activity of p85 was shown to occur in a mTORC1-independent manner, the enhanced proteasome-mediated degradation of phosphorylated p85 might interfere with this activity.

The different modalities of regulation displayed by the two isoforms of S6 kinase could be reflected by their different pattern of subcellular distribution. Previous studies suggested that the presence of a nuclear localization signal in the extended N-terminus of p85 induces its nuclear accumulation (Reinhard *et al.*, 1994). However, their localizations appear to be dynamic, since p70 has been shown to translocate in the nucleus upon phosphorylation by mTORC1 in the G_1 phase of the cell cycle and cytoplasmic pools of p85 have also been identified (Rosner and Hengstschläger, 2011). So far, it is not clear in which subcellular compartment the degradation of p85 occurs, in addition, the mechanism involved in its nucleo-cytoplasmic shuttling is not known. An interesting possibility is that hScrib might regulate the stability of phosphorylated p85 also through the control of its subcellular localization. Thus, the expression of hScrib might contribute to the correct localization of p85 and p70 S6 kinase isoforms, and its loss would therefore potentially lead to the mislocalization of S6 kinase to cellular compartments where the kinase is normally degraded. Although the levels of p70 S6 kinase are not rescued upon proteasome inhibition, previous studies suggested that p70 is degraded through a caspase-dependent mechanism (Dhar *et al.*, 2009). Therefore these data suggest the intriguing possibility that the two isoforms of S6 kinase are regulated by different proteolytic pathways and that hScrib might play a role in modulating their levels of expression. However, whether hScrib might protect p70 S6 kinase from caspase cleavage needs still to be determined. A summary of the potential regulation of PI3K/mTORC1 pathway by E6, E7 and hScrib is shown in Figure 33.

Taken together, these studies define a new function for hScrib in affecting protein translation, most likely through the modulation of S6 kinase activity. These studies also highlight the role of E6 in

fine-tuning the levels of expression and function of its target proteins, such that optimal conditions for viral replication are attained. However, the stimulation of growth-promoting pathways ultimately predispose cells to the acquisition of tumorigenic capacities during malignant progression.

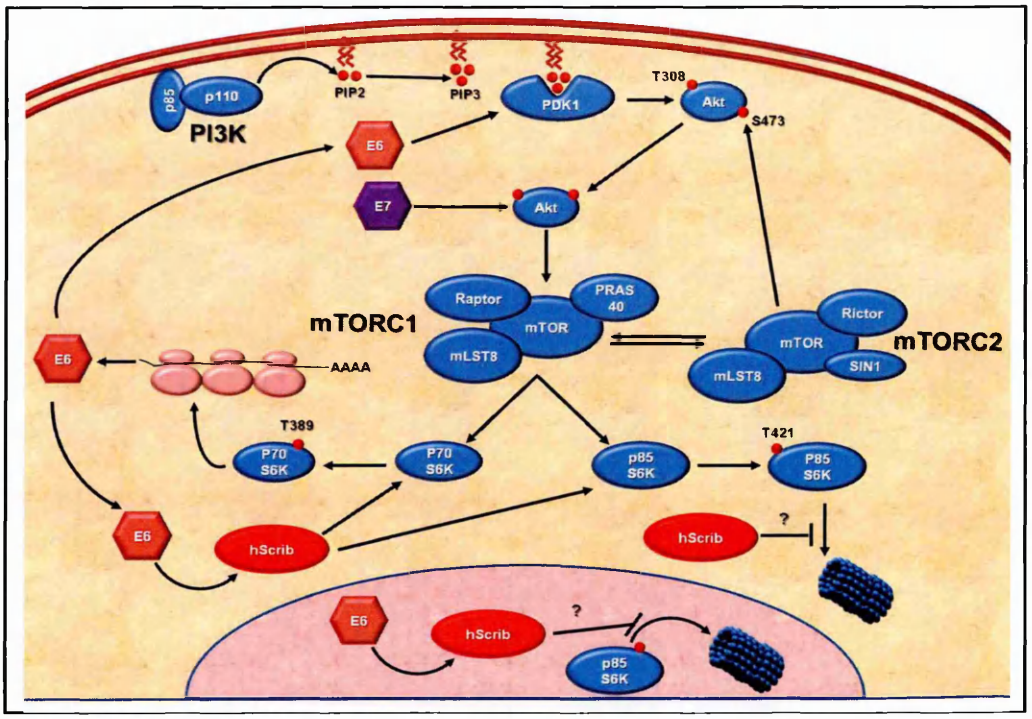

Figure 33. Regulation of the PI3K/mTORC1 pathway and protein translation by HPV oncoproteins and hScrib. Insulin or growth factor stimulation leads to the activation of the PI3K/mTORC1 pathway (see text). Both E6 and E7 have been shown to converge on the activation of this pathway through the regulation of Akt, and E6 may also stimulate Akt through the maintenance of high levels of PDK1 expression. E6 could also indirectly activate mTORC1 signaling, and protein translation, through the modulation of hScrib expression patterns leading to the upregulation of p70 and p85 S6 kinase isoforms. The ability of hScrib to regulate levels of p70 S6 kinase expression may then directly contribute to the expression of E6 through the regulation of its cap-dependent translation. The pattern of p85 S6 kinase appears to differ from that of the p70 isoform, and the mTORC-1 mediated phosphorylation may enhance its proteasome-mediated degradation. Apparently, the expression of hScrib and/or its modulation by E6 may counteract the degradation of p85. (PIP2, Phosphatidylinositol (3,4)-bisphosphate; PIP3, Phosphatidylinositol (3,4,5)-trisphosphate).

Plasmids

pCDNA-3 FLAG-tagged MAGI-1 has been described previously (Glaunsinger *et al.*, 2000). The K499E MAGI-1 mutant was generated using the GeneArt Site-Directed Mutagenesis System (Invitrogen) according to the manufacturer's instruction, using the following primers:

forward primer 5'TCCTGCAGATCGAAAGCCTCGTCCTCGATGGTCCT;

reverse primer 5'ACGAGGCTTTCGATCTGCAGGAACTCATCAGGCTC.

Untagged HPV-18 E6 and HPV-16 E6 pCDNA-3 expression plasmids have been described previously (Gardiol *et al.*, 1999; Pim *et al.*, 1994), as have the GST-fusion proteins HPV-18 E6 and HPV-16 E6 (Pim *et al.*,2000). The GST-fusion protein HPV-58 E6 was generated by subcloning PCR amplified HPV-58 E6 from the respective pcDNA construct, into compatible *Bam*H I and *Eco*R I restriction sites of pGEX2T using the following primers:

forward primer 5' ATGGATCCATGTTCCAGGACGCAGAG;

reverse primer 5' CGGAATTCTTACACTTGTGTTTGTCTGC.

pCMV MYC-tagged Net1 was described previously (García-Mata *et al.*, 2007) and HA-tagged β-catenin was kindly given by Prof. Claudio Brancolini.

Cell culture and transfection

HEK 293 (human embryonic kidney), U2OS (human osteosarcoma), HeLa (HPV-18-positive), CaSKi (HPV-16-positive), SiHa (HPV-16-positive), HaCaT (human immortalized keratinocytes) and H1299 (non-small cell lung carcinoma) cells were maintained in Dulbecco's modified Eagles Medium (DMEM) supplemented with 10% fetal bovine serum, penicillin-streptomycin (100 U/ml) and glutamine (300 μg/ml). For all siRNA (Dharmacon) delivery the cells were seeded on 6 cm dishes at a confluence of 1.2 x 10^5 and transfected using Lipofectamine 2000 (Invitrogen) with siRNA against luciferase, HPV-16 E6/E7 (5'UUAAAUGACAGCUCAGAGG), 18 E6/E7

(5'CAUUUACCAGCCCGACGAG), 18 E6 (5'CUCUGUGUAUGGAGACACA), E6AP or siRNA against the different PDZ-proteins (relevant Dharmacon Smart Pools). For siRNA transfection followed by immunofluorescent analysis, HeLa cells were seeded at a confluence of 1.2 x 10^5 on glass coverslips.

DNA transfection in HEK 293 cells was performed using the standard calcium phosphate precipitation protocol as described previously (Matlashewski *et al.*, 1987c). For DNA transfection in U2OS cells followed by immunofluorecent analysis, cells were seeded on glass coverslips at a confluence of 1.5 x 10^5 and transfected using the calcium phosphate precipitate protocol followed by glycerol shock. Briefly, 5 hours after the addition of the DNA precipitate, cells were treated with 15% glycerol in PBS for 1 minute. Cells were then washed for three times with 1X PBS and left to grow for additional 24 hours. For DNA transfection in HeLa, cells were seeded on 6 cm dishes at a confluence of 1.5 x 10^5 and transfected using Fugene HD (Promega). For DNA transfection followed by immunofluorescent analysis HeLa and HaCaT cells were seeded at the same confluence on glass coverslips.

Inhibitors

The proteasome inhibitor Z-leu-leu-leu-al (CBZ; Sigma) was dissolved in DMSO and used at 50 μM for the indicated time.

Antibodies

The following antibodies were used: mouse monoclonal anti-HA antibody 12CA5 (Roche), mouse monoclonal anti-ZO1 (ZO1-1A12) (Invitrogen), rabbit polyclonal anti-PAR3 (Millipore), mouse monoclonal anti-human pRB (BD Pharmingen). Mouse monoclonal anti-PSD95 (6G6-1C9) and mouse monoclonal anti-p84 (5E10) were from Abcam. The following antibodies were purchased from SIGMA: rabbit polyclonal anti-MAGI-1 (M5691), rabbit polyclonal anti-PTPN-3 (T6453) mouse monoclonal anti-α-tubulin (T6199), mouse monoclonal M2 anti-FLAG antibody (F3165) and rabbit polyclonal anti-FLAG (F7425). The following antibodies were purchased from Santa Cruz Biotechnology: mouse monoclonal anti-p53 (DO-1), mouse monoclonal anti-α-actinin (H-2),

mouse monoclonal anti-Dlg (2D11), goat polyclonal anti-Scribble (C-20), goat polyclonal anti-TIP2 (N-19), rabbit polyclonal anti-FAP-1 (H-300), rabbit polyclonal anti-E-cadherin (H-108), mouse monoclonal anti-Myc (9E10) and mouse monoclonal anti-vimentin (V-9). The following antibodies were from Cell Signaling Technology: rabbit polyclonal anti-Akt (9272), rabbit polyclonal anti-phosphorylated Akt (T308) (9275),rabbit polyclonal anti-phosphorylated Akt (S473) (9271), rabbit polyclonal anti-PDK1 (3062), rabbit monoclonal anti-phosphorylated PDK1 (S241) (C49H2), rabbit monoclonal anti-p70 S6 kinase (49D7), mouse monoclonal anti-phosphorylated p70 S6 kinase (T389) (1A5). The mouse monoclonal antibody anti HPV-18 E6 (N-terminus #399) was generated and generously provided by the Arbor Vita Corporation.

Western blotting and immunoprecipitation

For western blot sample preparation, cells were lysed in 2x SDS sample buffer (100mM Tris HCl pH 6.8; 200mM DTT, 4% SDS, 20% glycerol, 0.2% bromophenol blue) and the whole cell extracts were separated by SDS-PAGE and blotted on 0.22 nitrocellulose membranes (Schleicher and Schuell). The membranes were blocked at 37°C for 1 hour in 10% milk/PBS, except for those probed with anti-MAGI-1, anti-PTPN-3, and anti-PSD95 which were blocked in 5% milk/PBS; all membranes probed with the antibodies from Cell Signaling Technology were incubated in 5% milk/TBS 0.1% TWEEN 20; the membranes probed with the anti-HPV-18 E6 antibody were incubated in 2% BSA/5% milk in 1xTBS 0,1% TWEEN 20. The membranes were incubated with the appropriate primary antibodies diluted in 10% milk/PBS 0.5% TWEEN 20; except for the anti-MAGI-1, anti-PTPN-3 and anti-PSD95 antibodies which were diluted in 5% milk/PBS 0.05% TWEEN 20; all the antibodies from Cell Signaling Technology were diluted in 5% BSA/1xTBS 0.1% TWEEN except for the anti-phosphorylated p70 S6 kinase that was incubated in 5% milk/1xTBS 0.1% TWEEN 20. The HPV-18 E6 antibody was incubated in 1% BSA/2.5% milk in 1xTBS 0.1% TWEEN 20. The incubation times were 2 hours at room temperature for all antibodies, except for the anti-PTPN-3, anti-FAP1, anti-E-cadherin, anti-PSD95, all the Cell Signaling Technology antibodies and the anti-HPV-18 E6 antibody, which were incubated

overnight at 4°C. After several washes the membranes were incubated with the appropriate HRP-conjugated secondary antibody (DAKO) for 1 hour at room temperature. After extensive washing the blots were developed with ECL or ECL plus reagent (GE Healthcare) according to the manufacturer's instructions. Protein band intensities were quantitated where possible using the OptiQuant quantification program.

For co-immunoprecipitation experiments cells were scraped in ice cold PBS and extracted in lysis buffer (1% Triton X-100, 50mM Tris [pH 7.5], 300mM NaCl, 1mM EGTA, 1mM EDTA) supplemented with protease inhibitors (Set1, Calbiochem). The extracts were then passed through a 26G needle multiple times and then cleared by centrifugation. Extracts from cells expressing FLAG-tagged MAGI-1 constructs, were incubated with anti-FLAG beads (SIGMA) for 2 to 3 h on a rotating wheel at 4°C. For the immunoprecipitation of MYC-tagged NET1, cell extracts were incubated with the MYC antibody or the control antibody for approximately 3 hours on a rotating wheel at 4°C. Protein-A-Sepharose beads (GE Healthcare) were then added for an additional 60 minutes at 4°C. The beads were then extensively washed, and the immunoprecipitated proteins were analysed by western blotting.

Fusion protein purification and in vitro binding assays

GST-tagged fusion proteins were expressed and purified as described previously (Thomas *et al.*, 1996). Briefly, 40 ml of an overnight culture of *E.Coli* strain DH5-α previously transformed with the appropriate expression plasmids were inoculated into Luria Broth containing ampicillin (75μg/ml) and grown at 37°C up to an OD of 0.6 at 395 nm. Recombinant protein expression was induced for 3 hrs with 1mM isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma). The cells were harvested by centrifugation, disrupted by sonication in lysis buffer (1% Triton X-100 /1xPBS) and the lysates were cleared of cell debris by centrifugation. The GST-fusion proteins were then incubated for 1 hour with glutathione-conjugated agarose beads at 4°C. The purity of all fusion proteins was determined by SDS-PAGE and Coomassie Brilliant Blue R (Sigma) staining.

For *in vitro* binding and degradation assays, proteins were transcribed and translated *in vitro* in rabbit reticulocyte lysate using the Promega TNT system according to the manufacturer's instructions. The HPV-18 and HPV-16 E6 proteins were radiolabelled with [^{35}S]-cysteine while MAGI-1 proteins were radiolabelled with [^{35}S]-methionine.

Equal amounts of *in vitro*-translated proteins were added to GST fusion proteins bound to glutathione resin and incubated for 1 hour at 4°C. After extensive washing with PBS containing 0.5% NP-40, the bound proteins were analysed by SDS-PAGE and autoradiography.

For GST pull-down assays using cell extracts, FLAG-tagged wild type and K499E mutant MAGI-1 were transfected into HEK 293 cells. 24h after transfection cells were scraped in ice-cold PBS and extracted in lysis buffer (1% Triton X-100, 50mM Tris [pH 7.5], 300mM NaCl, 1mM EGTA, 1mM EDTA) supplemented with protease inhibitors (Set1, Calbiochem). The extracts were then passed through a 26G needle multiple times and cleared by centrifugation. Extracts from cells expressing FLAG-tagged MAGI-1 constructs, were incubated with the indicated GST-fusion proteins for 1-2 hours on a rotating wheel at 4°C. The beads were then extensively washed, and the immunoprecipitated proteins were analysed by western blotting.

In vivo degradation assays

HEK 293 cells were transfected with 1μg of either wild type or K499E MAGI-1 constructs along with 0.3 μg of LacZ. pCDNA3 18 E6 plasmid was also included at increasing concentrations: 2, 5, 10μg. 24 hours post-transfection, the cells were harvested and analyzed by western blotting.

In vitro degradation assays

Degradation assays were performed as previously described (Thomas *et al.*, 2001). Briefly, radiolabelled proteins were mixed and incubated for the indicated times at 30°C. Volumes were adjusted using water-primed lysate. The residual MAGI-1 proteins were analyzed by SDS-PAGE and autoradiography.

Subcellular fractionation assays

HeLa cells were seeded on 6 cm dishes and transfected with the relevant siRNAs. After 72h, cells were removed from the dishes by trypsinization and differential extraction of HeLa cells was performed to obtain cytoplasmic, membrane, nuclear and cytoskeletal fractions using the ProteoExtract Fractionation Kit (Calbiochem) according to the manufacturer's instructions. The differential protein expression plus fraction-specific markers, was analysed by SDS-PAGE western blotting.

Immunofluoresence microscopy and EdU staining

Cells were fixed with 3.7% paraformaldehyde in PBS for 20 minutes and permeabilized with 0.1% Triton X-100 in PBS for 5 minutes. Slides were incubated with primary antibodies for 2 hours at 37°C, extensively washed in PBS and incubated for 30 minutes at 37°C with secondary anti-rabbit or anti-mouse antibody conjugated to fluorescein or rhodamine (Molecular Probes). Samples were washed several times with water and mounted with Vectashield mounting medium (Vector Laboratories) on glass slides. Slides were analysed with either a Leica DMLB fluorescence microscope with a Leica photo camera (A01M871016), or a Zeiss LSM 510 confocal microscope with two lasers giving excitation lines at 480 and 510 nm. The data were collected with a 60x objective oil-immersion lens.

For EdU staining, cells were seeded on glass coverslips and transfected with FLAG-tagged wild type or K499E mutant MAGI-1 constructs. 24 hours after transfection EdU was added to the culture medium at a final concentration of 20μM for 2 hours. After labeling, cells were fixed with 3.7% paraformaldehyde in PBS for 20 minutes and permeabilized with 0.1% Triton X-100 in PBS for 5 minutes. Primary antibodies were incubated for 2 hours at 37°C, extensively washed in PBS and incubated for 30 minutes at 37°C with secondary anti-rabbit or anti-mouse antibody conjugated to fluorescein or rhodamine (Molecular Probes). After several washes in PBS, incorporated EdU was detected by incubating coverslips with the reaction mix solution (5mM (+)Na-L-Ascorbate (Sigma), 1mM copper sulphate, 0,05mM 6-Carboxyfluorescein-TEG azide) for 30 minutes at room temperature. Samples were washed several times with water and mounted with Vectashield

mounting medium (Vector Laboratories) on glass slides. Slides were analysed with Zeiss LSM 510 confocal microscope with two lasers giving excitation lines at 480 and 510 nm. The data were collected with a 60x objective oil-immersion lens.

TUNEL assays

Cells were seeded on glass coverslips and transfected with FLAG-tagged wild type or K499E mutant MAGI-1 constructs. 24 hours after transfection, were fixed with 3.7% paraformaldehyde in PBS for 20 minutes and permeabilized with 1xPBS 0.1% sodium citrate, 0.1% Triton X-100 for 5 minutes. The coverslips were incubated with the rabbit polyclonal FLAG antibody for 2 hours at 37°C, followed by incubation with the rhodamine-conjucated anti-rabbit antibody (Molecular Probes) for 30 minutes at 37°C. After several washes in PBS, apoptotic cells were detected using the fluorescein-conjugated *in situ* cell death detection kit (Roche) according to the manufacturer's instructions. Samples were washed several times with water and mounted with Vectashield mounting medium (Vector Laboratories) on glass slides. Slides were analysed with a Zeiss LSM 510 confocal microscope with two lasers giving excitation lines at 480 and 510 nm. The data were collected with a 60x objective oil-immersion lens.

Half-life experiments

72h post transfection, cells were treated for different time points as indicated with cycloheximide (50μg/ml in DMSO) to block protein synthesis. DMSO treated cells were used as the control. Total cellular extracts were then analyzed by Western blot and the intensity of the bands was measured using Optiquant program. The standard deviation was calculated from three independent assays.

Determination of HPV-18 E6 and p53 translation efficiency

HeLa cells were seeded on 6cm dishes at a confluence of 1.2 x $10^5$and transfected using Lipofectamine 2000 (Invitrogen) with siRNA against Luciferase or hScrib (Dharmacon). 72h after transfection cells were treated with cycloheximide (50μg/ml in DMSO) for additional 6 hours. Cells treated with DMSO alone were used as control for the expression of HPV-18 E6 and p53. In

order to monitor the recovery of E6 and p53 protein translation cycloheximide was removed and, after several washes in PBS, cells were left to grow for different time points in fresh DMEM supplemented with 10% fetal bovine serum, penicillin-streptomycin (100 U/ml) and glutamine (300 µg/ml). Total cellular extracts were prepared by harvesting cell in 2X SDS sample buffer, and then analyzed by Western blotting.

References

Abaan, O.D. *et al.*, 2005. PTPL1 is a direct transcriptional target of EWS-FLI1 and modulates Ewing's Sarcoma tumorigenesis. *Oncogene*, 24, pp.2715–2722.

Abaan, O.D. & Toretsky, J.A., 2008. PTPL1: a large phosphatase with a split personality. *Cancer metastasis reviews*, 27, pp.205–214.

Aijaz, S. *et al.*, 2005. Binding of GEF-H1 to the tight junction-associated adaptor cingulin results in inhibition of Rho signaling and G1/S phase transition. *Developmental Cell*, 8, pp.777–786.

Akerman, G.S. *et al.*, 2001 Human papillomavirus type 16 E6 and E7 cooperate to increase epidermal growth factor receptor (EGFR) mRNA levels, overcoming mechanisms by which excessive EGFR signaling shortens the life span of normal human keratinocytes. *Cancer Research*, 61, pp.3837-3843.

Alessi, D.R. *et al.*, 1996. Mechanism of activation of protein kinase B by insulin and IGF-1. *the The European Molecular Biology Organization Journal*, 15, pp.6541–6551.

Alewine, C. *et al.*, 2006. TIP-1 has PDZ scaffold antagonist activity. *Molecular Biology of the Cell*, 17, pp.4200–4211.

Alonso, A. *et al.*, 2004. Protein tyrosine phosphatases in the human genome. *Cell*, 117, pp.699–711.

Alvarez-Salas, L.M. *et al.*, 1998. Inhibition of HPV-16 E6/E7 immortalization of normal keratinocytes by hairpin ribozymes. *Proceedings of the National Academy of Sciences of the United States of America*, 95, pp.1189–1194.

Ando-Akatsuka, Y. *et al.*, 1999. Differential behavior of E-cadherin and occludin in their colocalization with ZO-1 during the establishment of epithelial cell polarity. *Journal of Cellular Physiology*, 179, pp.115–125.

Ankerst, J. & Jonsson, N., 1989. Adenovirus type 9-induced tumorigenesis in the rat mammary gland related to sex hormonal state. *Journal Of The National Cancer Institute*, 81, pp.294–298.

Aranda, V. *et al.*, 2006. Par6-aPKC uncouples ErbB2 induced disruption of polarized epithelial organization from proliferation control. *Nature Cell Biology*, 8, pp.1235–1245.

Aranda, V., Nolan, M.E. & Muthuswamy, S.K., 2008. Par complex in cancer: a regulator of normal cell polarity joins the dark side. *Oncogene*, 27, pp.6878–6887.

Arbeit, J.M. *et al.*, 1994. Progressive squamous epithelial neoplasia in K14-human papillomavirus type 16 transgenic mice. *Journal of Virology*, 68, pp.4358–4368.

Assefa, Z. *et al.*, 1999. The activation of the c-Jun N-terminal kinase and p38 mitogen-activated protein kinase signaling pathways protects HeLa cells from apoptosis following photodynamic therapy with hypericin. *The Journal of Biological Chemistry*, 274, pp.8788–96.

Badaracco, G. *et al.*, 2002. HPV16 and HPV18 in genital tumors: Significantly different levels of viral integration and correlation to tumor invasiveness. *Journal of Medical Virology*, 67, pp.574–582.

Bagchi, S., Raychaudhuri, P. & Nevins, J.R., 1990. Adenovirus E1A proteins can dissociate heteromeric complexes involving the E2F transcription factor: a novel mechanism for E1A trans-activation. *Cell*, 62, pp.659–669.

Baker, S.J. *et al.*, 1989. Chromosome 17 deletions and p53 gene mutations in colorectal carcinomas. *Science*, 244, pp.217–221.

Balda, M.S., Garrett, M.D. & Matter, K., 2003. The ZO-1-associated Y-box factor ZONAB regulates epithelial cell proliferation and cell density. *The Journal of Cell Biology*, 160, pp.423–432.

Balda, M.S. & Matter, K., 2000. The tight junction protein ZO-1 and an interacting transcription factor regulate ErbB-2 expression. *the The European Molecular Biology Organization Journal*, 19, pp.2024–2033.

Barbosa, M.S. & Schlegel, R., 1989. The E6 and E7 genes of HPV-18 are sufficient for inducing two-stage in vitro transformation of human keratinocytes. *Oncogene*, 4, pp.1529–1532.

Bauer, H. *et al.*, 2010. The Dual Role of Zonula Occludens (ZO) Proteins. *Journal of biomedicine biotechnology*, 2010, p.402593.

Bazzoni, G. *et al.*, 2000. Interaction of junctional adhesion molecule with the tight junction components ZO-1, cingulin, and occludin. *The Journal of Biological Chemistry*, 275, pp.20520–20526.

Bélanger, C. *et al.*, 2001. Human herpesvirus 8 viral FLICE-inhibitory protein inhibits Fas-mediated apoptosis through binding and prevention of procaspase-8 maturation. *Journal of Human Virology*, 4, pp.62–73.

Bernard, H.-U. *et al.*, 2010. Classification of papillomaviruses (PVs) based on 189 PV types and proposal of taxonomic amendments. *Virology*, 401, pp.70–79.

Bieging, K.T. & Attardi, L.D., 2012. Deconstructing p53 transcriptional networks in tumor suppression. *Trends in Cell Biology*, 22, pp.97–106.

Bilder, D., Li, M. & Perrimon, N., 2000. Cooperative regulation of cell polarity and growth by Drosophila tumor suppressors. *Science*, 289, pp.113–116.

Blobe, G.C. *et al.*, 2001. A novel mechanism for regulating transforming growth factor beta (TGF-beta) signaling. Functional modulation of type III TGF-beta receptor expression through interaction with the PDZ domain protein, GIPC. *The Journal of Biological Chemistry*, 276, pp.39608–39617.

Bodily, J. & Laimins, L.A., 2011. Persistence of human papillomavirus infection: keys to malignant progression. *Trends in Microbiology*, 19, pp.33–39.

Boon, S.S. & Banks, L., 2013. High-risk human papillomavirus E6 oncoproteins interact with 14-3-3ζ in a PDZ binding motif-dependent manner. *Journal of virology*, 87, pp.1586–95.

Bordin, M. *et al.*, 2004. Histone deacetylase inhibitors up-regulate the expression of tight junction proteins. *Molecular cancer research MCR*, 2, pp.692–701.

Bosch, F.X. *et al.*, 1992. Second International Workshop on the Epidemiology of Cervical Cancer and Human Papillomaviruses. *International Journal of Cancer*, 52, pp.171-173.

Boshart, M. *et al.*, 1984. A new type of papillomavirus DNA, its presence in genital cancer biopsies and in cell lines derived from cervical cancer. *the The European Molecular Biology Organization Journal*, 3, pp.1151–1157.

Bouvard, V. *et al.*, 1994. The human papillomavirus type 16 E5 gene cooperates with the E7 gene to stimulate proliferation of primary cells and increases viral gene expression. *Virology*, 203, pp.73–80.

Boyer, S.N., Wazer, D.E. & Band, V., 1996. E7 protein of human papilloma virus-16 induces degradation of retinoblastoma protein through the ubiquitin-proteasome pathway. *Cancer Research*, 56, pp.4620–4624.

Brandner, J.M. *et al.*, 2002. Organization and formation of the tight junction system in human epidermis and cultured keratinocytes. *European Journal of Cell Biology*, 81, pp.253–263.

Brehm, A. *et al.*, 1999. The E7 oncoprotein associates with Mi2 and histone deacetylase activity to promote cell growth. *the The European Molecular Biology Organization Journal*, 18, pp.2449–2458.

Butz, K. *et al.*, 2003. siRNA targeting of the viral E6 oncogene efficiently kills human papillomavirus-positive cancer cells. *Oncogene*, 22, pp.5938–5945.

Cao, T.T. *et al.*, 1999. A kinase-regulated PDZ-domain interaction controls endocytic sorting of the beta2-adrenergic receptor. *Nature*, 401, pp.286–290.

Capaldo, C.T. *et al.*, 2011. Tight function zonula occludens-3 regulates cyclin D1–dependent cell proliferation. *Molecular Biology of the Cell*, 22, pp.1677–1685.

Casamayor, A., Morrice, N.A. & Alessi, D.R., 1999. Phosphorylation of Ser-241 is essential for the activity of 3-phosphoinositide-dependent protein kinase-1: identification of five sites of phosphorylation in vivo. *The Biochemical journal*, 342, pp.287–292.

Cavatorta, A.L. *et al.*, 2004. Differential expression of the human homologue of Drosophila discs large oncosuppressor in histologic samples from human papillomavirus-associated lesions as a marker for progression to malignancy. *International journal of cancer Journal international du cancer*, 111, pp.373–380.

Chao, J.C.J. *et al.*, 2003. Effect of oral epidermal growth factor on mucosal healing in rats with duodenal ulcer. *World Journal of Gastroenterology*, 9, pp.2261–2265.

Charette, S.T. & McCance, D.J., 2007. The E7 protein from human papillomavirus type 16 enhances keratinocyte migration in an Akt-dependent manner. *Oncogene*, 26, pp.7386–7390.

Chaussepied, M. & Ginsberg, D., 2004. Transcriptional regulation of AKT activation by E2F. *Molecular Cell*, 16, pp.831–837.

Chen, E.Y. *et al.*, 1982. The primary structure and genetic organization of the bovine papillomavirus type 1 genome. *Nature*, 299, pp.529–534.

Chen, X. & Macara, I.G., 2005. Par-3 controls tight junction assembly through the Rac exchange factor Tiam1. *Nature Cell Biology*, 7, pp.262–269.

Cheng, J. *et al.*, 2002. A Golgi-associated PDZ domain protein modulates cystic fibrosis transmembrane regulator plasma membrane expression. *The Journal of Biological Chemistry*, 277, pp.3520–3529.

Cheng, J., Wang, H. & Guggino, W.B., 2004. Modulation of mature cystic fibrosis transmembrane regulator protein by the PDZ domain protein CAL. *The Journal of Biological Chemistry*, 279, pp.1892–1898.

Cheng, H. & Force, T., 2010. Molecular mechanisms of cardiovascular toxicity of targeted cancer therapeutics. *Circulation Research*, 106, pp.21–34.

Citi, S. *et al.*, 2009. The tight junction protein cingulin regulates gene expression and RhoA signaling. *Annals Of The New York Academy Of Sciences*, 1165, pp.88–98.

Collins, J.A. *et al.*, 1997. Major DNA fragmentation is a late event in apoptosis. *The journal of histochemistry and cytochemistry official journal of the Histochemistry Society*, 45, pp.923–934.

Conrad, M., Bubb, V.J. & Schlegel, R., 1993. The human papillomavirus type 6 and 16 E5 proteins are membrane-associated proteins which associate with the 16-kilodalton pore-forming protein. *Journal of Virology*, 67, pp.6170-6178.

Conrad, M. *et al.*, 1994. The E5 protein of HPV-6, but not HPV-16, associates efficiently with cellular growth factor receptors. *Virology*, 200, pp.796–800.

Cordenonsi, M. *et al.*, 2011. The Hippo Transducer TAZ Confers Cancer Stem Cell-Related Traits on Breast Cancer Cells. *Cell*, 147, pp.759–772.

Crusius, K. *et al.*, 1998. The human papillomavirus type 16 E5-protein modulates ligand-dependent activation of the EGF receptor family in the human epithelial cell line HaCaT. *Experimental Cell Research*, 241, pp.76–83.

Crusius, K., Rodriguez, I. & Alonso, A., 2000. The human papillomavirus type 16 E5 protein modulates ERK1/2 and p38 MAP kinase activation by an EGFR-independent process in stressed human keratinocytes. *Virus Genes*, 20, pp.65–69.

Cullen, A.P. *et al.*, 1991. Analysis of the physical state of different human papillomavirus DNAs in intraepithelial and invasive cervical neoplasm. *Journal of Virology*, 65, pp.606–612.

Cuppen, E. *et al.*, 2000. The zyxin-related protein TRIP6 interacts with PDZ motifs in the adaptor protein RIL and the protein tyrosine phosphatase PTP-BL. *European Journal of Cell Biology*, 79, pp.283–293.

D'Costa, Z.J. *et al.*, 2012. Transcriptional repression of E-cadherin by human papillomavirus type 16 E6. *PloS one*, 7, p.e48954.

Dajee, M. *et al.*, 2002. Epidermal Ras blockade demonstrates spatially localized Ras promotion of proliferation and inhibition of differentiation. *Oncogene*, 21, pp.1527–1538.

Danos, O., Katinka, M. & Yaniv, M., 1982. Human papillomavirus 1a complete DNA sequence: a novel type of genome organization among papovaviridae. *the The European Molecular Biology Organization Journal*, 1, pp.231–236.

Davis, M.A., Ireton, R.C. & Reynolds, A.B., 2003. A core function for p120-catenin in cadherin turnover. *The Journal of Cell Biology*, 163, pp.525–534.

Davy, C.E. *et al.*, 2006. HPV16 E1--E4 protein is phosphorylated by Cdk2/cyclin A and relocalizes this complex to the cytoplasm. *Virology*, 349, pp.230–244.

Davy, C.E. *et al.*, 2005. Human papillomavirus type 16 E1 E4-induced G2 arrest is associated with cytoplasmic retention of active Cdk1/cyclin B1 complexes. *Journal of Virology*, 79, pp.3998–4011.

DeCaprio, J.A. *et al.*, 1988. SV40 large tumor antigen forms a specific complex with the product of the retinoblastoma susceptibility gene. *Cell*, 54, pp.275–283.

Defilippis, R.A. *et al.*, 2003. Endogenous Human Papillomavirus E6 and E7 Proteins Differentially Regulate Proliferation , Senescence , and Apoptosis in HeLa Cervical Carcinoma Cells. *Society*, 77, pp.1551–1563.

Deng, W. *et al.*, 2004. Cyclin/CDK Regulates the Nucleocytoplasmic Localization of the Human Papillomavirus E1 DNA Helicase. *Journal of Virology*, 78, pp.13954–13965.

Denker, B.M. & Nigam, S.K., 1998. Molecular structure and assembly of the tight junction. *American Journal of Physiology*, 274, pp.F1–F9.

De Geest, K. *et al.*, 1993. Growth and differentiation of human papillomavirus type 31b positive human cervical cell lines. *Gynecologic Oncology*, 49, pp.303–310.

Delury, C.P. *et al.*, 2013. The role of protein kinase A regulation of the E6 PDZ-binding domain during the differentiation-dependent life cycle of human papillomavirus type 18. *Journal of virology*.

De Vries, L. *et al.*, 1998. GIPC, a PDZ domain containing protein, interacts specifically with the C terminus of RGS-GAIP. *Proceedings of the National Academy of Sciences of the United States of America*, 95, pp.12340–12345.

Der, C.J., Krontiris, T.G. & Cooper, G.M., 1982. Transforming genes of human bladder and lung carcinoma cell lines are homologous to the ras genes of Harvey and Kirsten sarcoma viruses. *Proceedings of the National Academy of Sciences of the United States of America*, 79, pp.3637–3640.

Dhar, R. *et al.*, 2009. Proteolytic cleavage of p70 ribosomal S6 kinase by caspase-3 during DNA damage-induced apoptosis. *Biochemistry*, 48, pp.1474–1480.

Dixon, E.P. *et al.*, 2000. The E1 helicase of human papillomavirus type 11 binds to the origin of replication with low sequence specificity. *Virology*, 270, pp.345–357.

Dobrosotskaya, I., Guy, R.K. & James, G.L., 1997. MAGI-1, a membrane-associated guanylate kinase with a unique arrangement of protein-protein interaction domains. *The Journal of Biological Chemistry*, 272, pp.31589–31597.

Dobrosotskaya, I.Y. & James, G.L., 2000. MAGI-1 interacts with beta-catenin and is associated with cell-cell adhesion structures. *Biochemical and Biophysical Research Communications*, 270, pp.903-909.

Dobrosotskaya, I.Y., 2001. Identification of mNET1 as a candidate ligand for the first PDZ domain of MAGI-1. *Biochemical and Biophysical Research Communications*, 283, pp.969–975.

Doorbar, J. *et al.*, 1997. Characterization of events during the late stages of HPV16 infection in vivo using high-affinity synthetic Fabs to E4. *Virology*, 238, pp.40–52.

Doorbar, J. *et al.*, 1991. Specific interaction between HPV-16 E1-E4 and cytokeratins results in collapse of the epithelial cell intermediate filament network. *Nature*, 352, pp.824–827.

Doorbar, J. *et al.*, 2012. The biology and life-cycle of human papillomaviruses. *Vaccine*, 30 Suppl 5, pp.F55–70.

Dow, L.E. *et al.*, 2003. hScrib is a functional homologue of the Drosophila tumour suppressor Scribble. *Oncogene*, 22, pp.9225–9230.

Dow, L.E. *et al.*, 2008. Loss of human Scribble cooperates with H-Ras to promote cell invasion through deregulation of MAPK signalling. *Oncogene*, 27, pp.5988–6001.

Doyle, D.A. *et al.*, 1996. Crystal structures of a complexed and peptide-free membrane protein-binding domain: molecular basis of peptide recognition by PDZ. *Cell*, 85, pp.1067–1076.

Dubash, A.D. *et al.*, 2011. The Small GTPase RhoA Localizes to the Nucleus and Is Activated by Net1 and DNA Damage Signals. *PLoS ONE*, 6, p.10.

Dürst, M. *et al.*, 1983. A papillomavirus DNA from a cervical carcinoma and its prevalence in cancer biopsy samples from different geographic regions. *Proceedings of the National Academy of Sciences of the United States of America*, 80, pp.3812–3815.

Dürst, M. *et al.*, 1987. Molecular and cytogenetic analysis of immortalized human primary keratinocytes obtained after transfection with human papillomavirus type 16 DNA. *Oncogene*, 1, pp.251–256.

Dyson, N. *et al.*, 1992. Homologous sequences in adenovirus E1A and human papillomavirus E7 proteins mediate interaction with the same set of cellular proteins. *Journal of Virology*, 66, pp.6893–6902.

Dyson, N. *et al.*, 1989. The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. *Science*, 243, pp.934–937.

Eastburn, D.J., Zegers, M.M. & Mostov, K.E., 2012. Scrib regulates HGF-mediated epithelial morphogenesis and is stabilized by Sgt1-HSP90. *Journal of Cell Science*.

Ebnet, K. *et al.*, 2000. Junctional adhesion molecule interacts with the PDZ domain-containing proteins AF-6 and ZO-1. *The Journal of Biological Chemistry*, 275, pp.27979–27988.

Ebnet, K. *et al.*, 2004. Junctional adhesion molecules (JAMs): more molecules with dual functions? *Journal of Cell Science*, 117, pp.19–29.

Ebnet, K. *et al.*, 2001. The cell polarity protein ASIP/PAR-3 directly associates with junctional adhesion molecule (JAM). *The EMBO journal*, 20, pp.3738–48.

Ebnet, K. *et al.*, 2003. The junctional adhesion molecule (JAM) family members JAM-2 and JAM-3 associate with the cell polarity protein PAR-3: a possible role for JAMs in endothelial cell polarity. *Journal of Cell Science*, 116, pp.3879–3891.

Eddy, B.E. *et al.*, 1961. Tumors induced in hamsters by injection of rhesus monkey kidney cell extracts. *Proceedings of the Society for Experimental Biology and Medicine*, 107, pp. 191-197.

Eddy, B.E. *et al.*, 1962. Identification of the oncogenic substance in rhesus monkey kidney cell culture as simian virus 40. Virology, 17, pp. 65–75.

Eichholtz-Wirth, H. & Sagan, D., 2000. IkappaB/NF-kappaB mediated cisplatin resistance in HeLa cells after low-dose gamma-irradiation is associated with altered SODD expression. *Apoptosis an international journal on programmed cell death*, 5, pp.255–263.

el-Deiry, W.S. *et al.*, 1993. WAF1, a potential mediator of p53 tumor suppression. *Cell*, 75, pp.817–825.

Erdmann, K.S. *et al.*, 2000. The Adenomatous Polyposis Coli-protein (APC) interacts with the protein tyrosine phosphatase PTP-BL via an alternatively spliced PDZ domain. *Oncogene*, 19, pp.3894–3901.

Etienne-Manneville, S. *et al.*, 2005. Cdc42 and Par6–PKCζ regulate the spatially localized association of Dlg1 and APC to control cell polarization. *The Journal of Cell Biology*, 170, pp.895–901.

Fang, Q.I. *et al.*, 2011. Simian virus 40 transformation, malignant mesothelioma and brain tumors. *Expert Review of Respiratory Medicine*, 5, pp.683-697.

Farkas, A.E., Capaldo, C.T. & Nusrat, A., 2012. Regulation of epithelial proliferation by tight junction proteins. *Annals Of The New York Academy Of Sciences*, 1258, pp.115–24.

Favre-Bonvin, A. *et al.*, 2005. Human Papillomavirus Type 18 E6 Protein Binds the Cellular PDZ Protein TIP-2/GIPC, Which Is Involved in Transforming Growth Factor β Signaling and Triggers Its Degradation by the Proteasome. *Journal of Virology*, 79, pp.4229–4237.

Fehrmann, F., Klumpp, D.J. & Laimins, L.A., 2003. Human papillomavirus type 31 E5 protein supports cell cycle progression and activates late viral functions upon epithelial differentiation. *Journal of Virology*, 77, pp.2819–2831.

Felsani, A., Mileo, A.M. & Paggi, M.G., 2006. Retinoblastoma family proteins as key targets of the small DNA virus oncoproteins. *Oncogene*, 25, pp.5277–5285.

Feng, H. *et al.*, 2008. Clonal integration of a polyomavirus in human Merkel cell carcinoma. *Science*, 319, pp.1096–1100.

Ferber, E.C. *et al.*, 2008. A role for the cleaved cytoplasmic domain of E-cadherin in the nucleus. *The Journal of Biological Chemistry*, 283, pp.12691–12700.

Fingar, D.C. & Blenis, J., 2004. Target of rapamycin (TOR): an integrator of nutrient and growth factor signals and coordinator of cell growth and cell cycle progression. *Oncogene*, 23, pp.3151–71.

Finlay, C.A., Hinds, P.W. & Levine, A.J., 1989. The p53 proto-oncogene can act as a suppressor of transformation. *Cell*, 57, pp.1083–93.

Foehr, E.D. *et al.*, 2005. FAS associated phosphatase (FAP-1) blocks apoptosis of astrocytomas through dephosphorylation of FAS. *Journal of neurooncology*, 74, pp.241–248.

Forman, D. *et al.*, 2012. Global burden of human papillomavirus and related diseases. *Vaccine*, 30, pp.F12–F23.

Fournane, S. *et al.*, 2011. Surface plasmon resonance analysis of the binding of high-risk mucosal HPV E6 oncoproteins to the PDZ1 domain of the tight junction protein MAGI-1. *Journal of molecular recognition JMR*, 24, pp.511–523.

Freedman, D.A. & Levine, A.J., 1998. Nuclear Export Is Required for Degradation of Endogenous p53 by MDM2 and Human Papillomavirus E6. *Molecular and Cellular Biology*, 18, pp.7288–7293.

Frese, K.K. *et al.*, 2006. Oncogenic function for the Dlg1 mammalian homolog of the Drosophila discs-large tumor suppressor. *the The European Molecular Biology Organization Journal*, 25, pp.1406–1417.

Frese, K.K. *et al.*, 2003. Selective PDZ protein-dependent stimulation of phosphatidylinositol 3-kinase by the adenovirus E4-ORF1 oncoprotein. *Oncogene*, 22, pp.710–721.

Funk, J.O. *et al.*, 1997. Inhibition of CDK activity and PCNA-dependent DNA replication by p21 is blocked by interaction with the HPV-16 E7 oncoprotein. *Genes & Development*, 11, pp.2090–2100.

García-Mata, R. *et al.*, 2007. The Nuclear RhoA Exchange Factor Net1 Interacts with Proteins of the Dlg Family, Affects Their Localization, and Influences Their Tumor Suppressor Activity. *Molecular and Cellular Biology*, 27, pp.8683–8697.

Gardiol, D. *et al.*, 1999. Oncogenic human papillomavirus E6 proteins target the discs large tumour suppressor for proteasome-mediated degradation. *Oncogene*, 18, pp.5487–5496.

Gardiol, D. *et al.*, 2006. Human discs large and scrib are localized at the same regions in colon mucosa and changes in their expression patterns are correlated with loss of tissue architecture during malignant progression. *International Journal of Cancer*, 119, 1285-1290.

Gebauer, F. & Hentze, M.W., 2004. Molecular mechanisms of translational control. *Nature Reviews Molecular Cell Biology*, 5, pp.827–835.

Genovese, N.J. *et al.*, 2008. Casein kinase II motif-dependent phosphorylation of human papillomavirus E7 protein promotes p130 degradation and S-phase induction in differentiated human keratinocytes. *Journal of Virology*, 82, pp.4862–4873.

Genther, S.M. *et al.*, 2003. Quantitative role of the human papillomavirus type 16 E5 gene during the productive stage of the viral life cycle. *Journal of Virology*, 77, pp.2832–2842.

Gentzsch, M. *et al.*, 2003. The PDZ-binding chloride channel ClC-3B localizes to the Golgi and associates with cystic fibrosis transmembrane conductance regulator-interacting PDZ proteins. *The Journal of Biological Chemistry*, 278, pp.6440–6449.

Giles, R.H., Van Es, J.H. & Clevers, H., 2003. Caught up in a Wnt storm: Wnt signaling in cancer. *Biochimica et Biophysica Acta*, 1653, pp.1–24.

Girardi, A.J. *et al.*, 1962. Development of tumors in hamsters inoculated in the neonatal period with vacuolating virus, SV-40. *Proceedings of the Society for Experimental Biology and Medicine*, 109, pp 649–660.

Glaunsinger, B.A. *et al.*, 2000. Interactions of the PDZ-protein MAGI-1 with adenovirus E4-ORF1 and high-risk papillomavirus E6 oncoproteins. *Oncogene*, 19, pp.5270–5280.

Glaunsinger, B.A. *et al.*, 2001. Link of the unique oncogenic properties of adenovirus type 9 E4-ORF1 to a select interaction with the candidate tumor suppressor protein ZO-2. *the The European Molecular Biology Organization Journal*, 20, pp.5578–5586.

Glondu-Lassis, M. *et al.*, 2010. PTPL1/PTPN13 regulates breast cancer cell aggressiveness through direct inactivation of Src kinase. *Cancer Research*, 70, pp.5116–5126.

Gonzalez, S.L. *et al.*, 2001. Degradation of the retinoblastoma tumor suppressor by the human papillomavirus type 16 E7 oncoprotein is important for functional inactivation and is separable from proteasomal degradation of E7. *J Virol*, 75, pp.7583–7591.

Gonzalez-Mariscal, L. *et al.*, 2009. The tight junction protein ZO-2 blocks cell cycle progression and inhibits cyclin D1 expression. *Annals Of The New York Academy Of Sciences*, 1165, pp.121–125.

Goodman, R.H. & Smolik, S., 2000. CBP/p300 in cell growth, transformation, and development. *Genes & Development*, 14, pp.1553–1577.

Gottardi, C.J. *et al.*, 1996. The junction-associated protein, zonula occludens-1, localizes to the nucleus before the maturation and during the remodeling of cell-cell contacts. *Proceedings of the National Academy of Sciences of the United States of America*, 93, pp.10779–10784.

Graham, F.L. *et al.*, 1984. Transformation by human adenoviruses. *Seminars in Cancer Biology*, 3, pp.151–163.

Gregorc, U. *et al.*, 2007. Cleavage of MAGI-1, a tight junction PDZ protein, by caspases is an important step for cell-cell detachment in apoptosis. *Apoptosis an international journal on programmed cell death*, 12, pp.343–354.

Grifoni, D. *et al.*, 2004. The human protein Hugl-1 substitutes for Drosophila lethal giant larvae tumour suppressor function in vivo. *Oncogene*, 23, pp.8688–8694.

Gross L., 1953. A filterable agent, recovered from Ak leukemic extracts, causing salivary gland carcinomas in C3H mice. *Proceedings of the Society for Experimental Biology and Medicine*, 83, pp. 414–21.

Grossman, S.R., Mora, R. & Laimins, L.A., 1989. Intracellular localization and DNA-binding properties of human papillomavirus type 18 E6 protein expressed with a baculovirus vector. *Journal of Virology*, 63, pp.366–374.

Halbert, D.N., Cutt, J.R. & Shenk, T., 1985. Adenovirus early region 4 encodes functions required for efficient DNA replication, late gene expression, and host cell shutoff. *Journal of Virology*, 56, pp.250–257.

Hampson, L. *et al.*, 2004. The PDZ protein Tip-1 is a gain of function target of the HPV16 E6 oncoprotein. *International Journal of Oncology*, 25, pp.1249–1256.

Han, J. & Sun, P., 2007. The pathways to tumor suppression via route p38. *Trends in Biochemical Sciences* , 32, pp.364–371.

Han, M. *et al.*, 2012. Expression of TIP-1 confers radioresistance of malignant glioma cells. *PloS one*, 7, p.e45402.

Han, X. *et al.*, 2012. Neuroepithelial transforming protein 1 short interfering RNA-mediated gene silencing with microbubble and ultrasound exposure inhibits the proliferation of hepatic carcinoma cells in vitro. *Journal of ultrasound in medicine official journal of the American Institute of Ultrasound in Medicine*, 31, pp.853–61.

Handa, K. *et al.*, 2007. E6AP-Dependent Degradation of DLG4/PSD95 by High-Risk Human Papillomavirus Type 18 E6 Protein. *Journal of Virology*, 81, pp.1379–1389.

Harris, B.Z. & Lim, W.A., 2001. Mechanism and role of PDZ domains in signaling complex assembly. *J Cell Sci*, 114, pp.3219–3231.

Hassel, B. *et al.*, 2003. CALEB/NGC interacts with the Golgi-associated protein PIST. *The Journal of Biological Chemistry*, 278, pp.40136–40143.

Hawley-Nelson, P. *et al.*, 1989. HPV16 E6 and E7 proteins cooperate to immortalize human foreskin keratinocytes. *the The European Molecular Biology Organization Journal*, 8, pp.3905–3910.

Hegedüs, T. *et al.*, 2003. C-terminal phosphorylation of MRP2 modulates its interaction with PDZ proteins. *Biochemical and Biophysical Research Communications*, 302, pp.454–461.

Helt, A.-M., Funk, J.O. & Galloway, D.A., 2002. Inactivation of both the retinoblastoma tumor suppressor and p21 by the human papillomavirus type 16 E7 oncoprotein is necessary to inhibit cell cycle arrest in human epithelial cells. *Journal of Virology*, 76, pp.10559–10568.

Herfs, M. *et al.*, 2012. A discrete population of squamocolumnar junction cells implicated in the pathogenesis of cervical cancer. *Proceedings of the National Academy of Sciences of the United States of America*, 109, pp. 10516–21.

Higuchi, M. *et al.*, 2007. Cooperation of NF-kappaB2/p100 activation and the PDZ domain binding motif signal in human T-cell leukemia virus type 1 (HTLV-1) Tax1 but not HTLV-2 Tax2 is crucial for interleukin-2-independent growth transformation of a T-cell line. *Journal of Virology*, 81, pp. 11900-11907.

Hilleman, M.R., Werner, J.H., 1954. Recovery of new agent from patients with acute respiratory illness. *Proceedings of the Society for Experimental Biology and Medicine, 85,* pp. 183-188.

Hinds, P., Finlay, C. & Levine, A.J., 1989. Mutation is required to activate the p53 gene for cooperation with the ras oncogene and transformation. *Journal of Virology*, 63, pp.739–746.

Hirabayashi, S. *et al.*, 2003. JAM4, a junctional cell adhesion molecule interacting with a tight junction protein, MAGI-1. *Molecular and Cellular Biology*, 23, pp.4267–4282.

Hirata, A. *et al.*, 2004. PDZ domain-binding motif of human T-cell leukemia virus type 1 Tax oncoprotein augments the transforming activity in a rat fibroblast cell line. *Virology*, 318, pp.327–336.

Hirose, T. *et al.*, 2002. Involvement of ASIP/PAR-3 in the promotion of epithelial tight junction formation. *Journal of Cell Science*, 115, pp.2485–2495.

Hoffmann, M. *et al.*, 2012. HPV DNA, E6*I-mRNA expression and p16(INK4A) immunohistochemistry in head and neck cancer - how valid is p16(INK4A) as surrogate marker? *Cancer Letters*, 323, pp.88–96.

Holmgren, S.C. *et al.*, 2005. The Minor Capsid Protein L2 Contributes to Two Steps in the Human Papillomavirus Type 31 Life Cycle. *Journal of Virology*, 79, pp.3938–3948.

Hoover, K.B., Liao, S.-Y. & Bryant, P.J., 1998. Loss of the tight junction MAGUK ZO-1 in breast cancer: relationship to glandular differentiation and loss of heterozygosity. *The American journal of pathology*, 153, pp.1767–1773.

Hou, S.-W. *et al.*, 2010. PTPH1 dephosphorylates and cooperates with p38gamma MAPK to increase ras oncogenesis through PDZ-mediated interaction. *Cancer Research*, 70, pp.2901–2910.

Houben. R., *et al.*, 2010. Merkel cell polyomavirus-infected Merkel cell carcinoma cells require expression of viral T antigens. *Journal of Virology*, 84, pp. 7064-7072.

Houweling, A., van den Elsen, P.J., van der Eb, A.J., 1980. Partial transformation of primary rat cells by the leftmost 4.5% fragment of adenovirus 5 DNA. *Virology*, 105, 537-550.

Hu, Y. *et al.*, 2009. HPV16 E6-induced and E6AP-dependent inhibition of the transcriptional coactivator hADA3 in human cervical carcinoma cells. *Cancer Investigation*, 27, pp.298–306.

Hu, Y. *et al.*, 2007. MAGI-2 Inhibits cell migration and proliferation via PTEN in human hepatocarcinoma cells. *Archives of Biochemistry and Biophysics*, 467, pp.1–9.

Huber, A.H. & Weis, W.I., 2001. The structure of the beta-catenin/E-cadherin complex and the molecular basis of diverse ligand recognition by beta-catenin. *Cell*, 105, pp.391–402.

Huibregtse, J.M., Scheffner, M. & Howley, P.M., 1993. Localization of the E6-AP regions that direct human papillomavirus E6 binding, association with p53, and ubiquitination of associated proteins. *Molecular and Cellular Biology*, 13, pp.4918–4927.

Hurd, T.W. *et al.*, 2003. Direct interaction of two polarity complexes implicated in epithelial tight junction assembly. *Nature cell biology*, 5, pp.137–42.

Hynes, N.E. & Lane, H.A., 2005. ERBB receptors and cancer: the complexity of targeted inhibitors. *Nature Reviews Cancer*, 5, pp.341–354.

Ilves, I. *et al.*, 2006. Brd4 Is Involved in Multiple Processes of the Bovine Papillomavirus Type 1 Life Cycle. *Journal of Virology*, 80, pp.3660–3665.

Itoh, M. *et al.*, 2001. Junctional adhesion molecule (JAM) binds to PAR-3. *The Journal of Cell Biology*, 154, pp.491–498.

Ivanov, V.N. *et al.*, 2003. FAP-1 Association with Fas (Apo-1) Inhibits Fas Expression on the Cell Surface. *Molecular and Cellular Biology*, 23, pp.3623–3635.

Ivanov, A.I. *et al.*, 2010. Tumor Suppressor Scribble Regulates Assembly of Tight Junctions in the Intestinal Epithelium. *The American journal of pathology*, 176, pp.134–145.

Ivanova, S. *et al.*, 2007. Cellular localization of MAGI-1 caspase cleavage products and their role in apoptosis. *Biological Chemistry*, 388, pp.1195–1198.

Izumi, Y. *et al.*, 1998. An atypical PKC directly associates and colocalizes at the epithelial tight junction with ASIP, a mammalian homologue of Caenorhabditis elegans polarity protein PAR-3. *The Journal of Cell Biology*, 143, pp.95–106.

Jabbar, S. *et al.*, 2010. Human papillomavirus type 16 E6 and E7 oncoproteins act synergistically to cause head and neck cancer in mice. *Virology*, 407, pp.60-67.

Jacinto, E. *et al.*, 2006. SIN1/MIP1 maintains rictor-mTOR complex integrity and regulates Akt phosphorylation and substrate specificity. *Cell*, 127, pp.125–137.

Jakob, T. & Udey, M.C., 1998. Regulation of E-cadherin-mediated adhesion in Langerhans cell-like dendritic cells by inflammatory mediators that mobilize Langerhans cells in vivo. *The Journal of Immunology*, 160, pp.4067–4073.

Javier, R.T. & Butel, J.S., 2008. The history of tumor virology. *Cancer Research*, 68, pp.7693–7706.

Javier, R.T., 2008. Cell polarity proteins: common targets for tumorigenic human viruses. *Oncogene*, 27, pp.7031–46.

Javier, R.T., Raska Jr., K. & Shenk, T., 1992. Requirement for the adenovirus type 9 E4 region in production of mammary tumors. *Science*, 257, pp.1267–1271.

Jefferies, H.B. *et al.*, 1997. Rapamycin suppresses 5'TOP mRNA translation through inhibition of p70s6k. *the The European Molecular Biology Organization Journal*, 16, pp.3693–3704.

Jeleń, F. *et al.*, 2003. PDZ domains - common players in the cell signaling. *Acta Biochimica Polonica*, 50, pp.985–1017.

Jemth, P. & Gianni, S., 2007. Current Topics PDZ Domains : Folding and Binding. *Biochemistry*, 46, pp.8701–8.

Jensen. F., Koprowski H., & Ponten J.A., 1963. Rapid transformation of human fibroblast cultures by simian virus. *Proceedings of the National Academy of Sciences of the United States of America*, 50, pp. 343-348.

Jeon, S., Allen-Hoffmann, B.L. & Lambert, P.F., 1995. Integration of human papillomavirus type 16 into the human genome correlates with a selective growth advantage of cells. *Journal of Virology*, 69, pp.2989–2997.

Jeong, K.W. *et al.*, 2007. Human papillomavirus type 16 E6 protein interacts with cystic fibrosis transmembrane regulator-associated ligand and promotes E6-associated protein-mediated ubiquitination and proteasomal degradation. *Oncogene*, 26, pp.487–499.

Jia, C.H. *et al.*, 2012. IKK-β mediates hydrogen peroxide induced cell death through p85 S6K1. *Cell death and differentiation*, pp.1–11.

Jiang, M. *et al.*, 2009. The role of polyomaviruses in human disease. *Virology*, 384, pp.266–273.

Jing, M. *et al.*, 2007. Degradation of Tyrosine Phosphatase PTPN3 (PTPH1) by Association with Oncogenic Human Papillomavirus E6 Proteins. *Journal of Virology*, 81, pp.2231–2239.

Joberty, G. *et al.*, 2000. The cell-polarity protein Par6 links Par3 and atypical protein kinase C to Cdc42. *NATURE CELL BIOLOGY*, 2, pp.531–539.

Johnson, M.H., 2009. From mouse egg to mouse embryo: polarities, axes, and tissues. *Annual Review of Cell and Developmental Biology*, 25, pp.483–512.

Jones, D.L., Thompson, D.A. & Münger, K., 1997. Destabilization of the RB tumor suppressor protein and stabilization of p53 contribute to HPV type 16 E7-induced apoptosis. *Virology*, 239, pp.97–107.

Ju, S.T. *et al.*, 1994. Participation of target Fas protein in apoptosis pathway induced by CD4+ Th1 and CD8+ cytotoxic T cells. *Proceedings of the National Academy of Sciences of the United States of America*, 91, pp.4185–4189.

Kabsch, K. *et al.*, 2004. The HPV-16 E5 protein inhibits TRAIL- and FasL-mediated apoptosis in human keratinocyte raft cultures. *Intervirology*, 47, pp.48–56.

Kämper, N. *et al.*, 2006. A membrane-destabilizing peptide in capsid protein L2 is required for egress of papillomavirus genomes from endosomes. *Journal of Virology*, 80, pp.759–768.

Kanodia, S., Fahey, L.M. & Kast, W.M., 2007. Mechanisms used by human papillomaviruses to escape the host immune response. *Current Cancer Drug Targets*, 7, pp.79–89.

Kao, W.H. *et al.*, 2000. Human Papillomavirus Type 16 E6 Induces Self-Ubiquitination of the E6AP Ubiquitin-Protein Ligase. *Journal of Virology*, 74, pp.6408–6417.

Kashyap, A. *et al.*, 2012. The human Lgl polarity gene, Hugl-2, induces MET and suppresses Snail tumorigenesis. *Oncogene*, pp.1–12.

Kastan, M.B. & Bartek, J., 2004. Cell-cycle checkpoints and cancer. *Nature*, 432, pp.316–323.

Kim, Y.S. *et al.*, 2002. The rgl-1 is a legitimate homologue of lethal giant larvae recessive oncogene in rat. *International Journal of Oncology*, 20, pp.1219–1225.

Kim, E. & Sheng, M., 2004. PDZ domain proteins of synapses. *Nature Reviews Neuroscience*, 5, pp.771–781.

Kimura, R. *et al.*, 2010. Interaction of endothelial cell-selective adhesion molecule and MAGI-1 promotes mature cell-cell adhesion via activation of RhoA. *Genes to cells devoted to molecular cellular mechanisms*, 15, pp.385–396.

Kinzler, K. & Vogelstein, B., 1996. Lessons from Hereditary Review Colorectal Cancer. *Cell.*

Kirnbauer, R. *et al.*, 1992. Papillomavirus L1 major capsid protein self-assembles into virus-like particles that are highly immunogenic. *Proceedings of the National Academy of Sciences of the United States of America*, 89, pp.12180–4.

Kirschner, N. & Brandner, J.M., 2012. Barriers and more: functions of tight junction proteins in the skin. *Annals Of The New York Academy Of Sciences*, 1257, pp.158–66.

Kiyono, T. *et al.*, 1997. Binding of high-risk human papillomavirus E6 oncoproteins to the human homologue of the Drosophila discs large tumor suppressor protein. *Proceedings of the National Academy of Sciences of the United States of America*, 94, pp.11612–11616.

Klingelhutz, A.J. *et al.*, 1994. Restoration of telomeres in human papillomavirus-immortalized human anogenital epithelial cells. *Molecular and Cellular Biology*, 14, pp.961–969.

Klingelhutz, A.J., Foster, S.A. & McDougall, J.K., 1996. Telomerase activation by the E6 gene product of human papillomavirus type 16. *Nature*, 380, pp.79–82.

Klingelhutz, A.J. & Roman, A., 2012. Cellular transformation by human papillomaviruses: Lessons learned by comparing high- and low-risk viruses. *Virology*, 424, pp.77–98.

Knight, G.L. *et al.*, 2004. Cooperation between different forms of the human papillomavirus type 1 E4 protein to block cell cycle progression and cellular DNA synthesis. *Society*, 78, pp.13920–13933.

Knight, G.L., Turnell, A.S. & Roberts, S., 2006. Role for Wee1 in Inhibition of G2-to-M Transition through the Cooperation of Distinct Human Papillomavirus Type 1 E4 Proteins. *Journal of Virology*, 80, pp.7416–7426.

Knoblich, J.A., 2008. Mechanisms of asymmetric stem cell division. *Cell*, 132, pp.583–597.

Kobayashi, S. *et al.*, 2005. 53BP2 induces apoptosis through the mitochondrial death pathway. *Genes to cells devoted to molecular cellular mechanisms*, 10, pp.253–260.

Kotelevets, L. *et al.*, 2005. Implication of the MAGI-1b/PTEN signalosome in stabilization of adherens junctions and suppression of invasiveness. *The FASEB journal official publication of the Federation of American Societies for Experimental Biology*, 19, pp.115–117.

Kozak, M., 1989. Context effects and inefficient initiation at non-AUG codons in eucaryotic cell-free translation systems. *Molecular and Cellular Biology*, 9, pp.5073–5080.

Kranjec, C. & Banks, L., 2011. A systematic analysis of human papillomavirus (HPV) E6 PDZ substrates identifies MAGI-1 as a major target of HPV type 16 (HPV-16) and HPV-18 whose loss accompanies disruption of tight junctions. *Journal of Virology*, 85, pp.1757-1764.

Krishna Subbaiah, V. *et al.*, 2012. The invasive capacity of HPV transformed cells requires the hDlg-dependent enhancement of SGEF/RhoG activity. *PLoS Pathogens*, 8, p.e1002543.

Kuang, S.Q. *et al.*, 2008. Genome-wide identification of aberrantly methylated promoter associated CpG islands in acute lymphocytic leukemia. *Leukemia official journal of the Leukemia Society of America Leukemia Research Fund UK*, 22, pp.1529–1538.

Lechler, T. & Fuchs, E., 2005. Asymmetric cell divisions promote stratification and differentiation of mammalian skin. *Nature*, 437, pp.275–280.

Lechner, M.S. *et al.*, 1992. Human papillomavirus E6 proteins bind p53 in vivo and abrogate p53-mediated repression of transcription. *the The European Molecular Biology Organization Journal*, 11, pp.3045–3052.

Lee, S.S., Weiss, R.S. & Javier, R.T., 1997. Binding of human virus oncoproteins to hDlg/SAP97, a mammalian homolog of the Drosophila discs large tumor suppressor protein. *Proceedings of the National Academy of Sciences of the United States of America*, 94, pp.6670–6675.

Lee, S.S. *et al.*, 2000. Multi-PDZ Domain Protein MUPP1 Is a Cellular Target for both Adenovirus E4-ORF1 and High-Risk Papillomavirus Type 18 E6 Oncoproteins. *Journal of Virology*, 74, pp.9680–9693.

Lee, C. & Laimins, L.A., 2004. Role of the PDZ Domain-Binding Motif of the Oncoprotein E6 in the Pathogenesis of Human Papillomavirus Type 31. *Society*, 78, pp.12366–12377.

Leechanachai, P. *et al.*, 1992. The E5 gene from human papillomavirus type 16 is an oncogene which enhances growth factor-mediated signal transduction to the nucleus. *Oncogene*, 7, pp.19–25.

Leslie, N.R. & Downes, C.P., 2002. PTEN: The down side of PI 3-kinase signalling. *Cellular Signalling*, 14, pp.285–295.

Levine, A.J., Finlay, C.A., & Hinds, P.W., 2004. P53 is a tumor suppressor gene. *Cell*, 116, (2 Suppl) S67-69, 1 p following S69.

Leyden, J. *et al.*, 2006. Net1 and Myeov: computationally identified mediators of gastric cancer. *British Journal of Cancer*, 94, pp.1204–1212.

Li, D. & Mrsny, R.J., 2000. Oncogenic Raf-1 disrupts epithelial tight junctions via downregulation of occludin. *The Journal of Cell Biology*, 148, pp.791–800.

Li, S. *et al.*, 1999. The human papilloma virus (HPV)-18 E6 oncoprotein physically associates with Tyk2 and impairs Jak-STAT activation by interferon-alpha. *Oncogene*, 18, pp.5727–5737.

Li, X. *et al.*, 2011. Scribble-mediated membrane targeting of PHLPP1 is required for its negative regulation of Akt. *EMBO Reports*, 12, pp.818–824.

Lin, D. *et al.*, 2000. A mammalian PAR-3-PAR-6 complex implicated in Cdc42/Rac1 and aPKC signalling and cell polarity. *Nature Cell Biology*, 2, pp.540–547.

Linzer, D.I. & Levine, A.J., 1979. Characterization of a 54K dalton cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells. *Cell*, 17, pp.43–52.

Linzer, D.I., Maltzman, W. & Levine, A.J., 1979. The SV40 A gene product is required for the production of a 54,000 MW cellular tumor antigen. *Virology*, 98, pp.308–318.

Lisovsky, M. *et al.*, 2009. Cell polarity protein Lgl2 is lost or aberrantly localized in gastric dysplasia and adenocarcinoma: an immunohistochemical study. *Modern pathology an official journal of the United States and Canadian Academy of Pathology Inc*, 22, pp.977–984.

Litovchick, L., Chestukhin, A. & DeCaprio, J.A., 2004. Glycogen Synthase Kinase 3 Phosphorylates RBL2/p130 during Quiescence. *Molecular and Cellular Biology*, 24, pp.8970–8980.

Liu, H. *et al.*, 2010. The ESEV PDZ-binding motif of the avian influenza A virus NS1 protein protects infected cells from apoptosis by directly targeting Scribble. *Journal of Virology*, 84, pp.11164–11174.

Liu, X. *et al.*, 2009. HPV E6 protein interacts physically and functionally with the cellular telomerase complex. *Proceedings of the National Academy of Sciences of the United States of America*, 106, pp.18780–18785.

Lohia, M., Qin, Y. & Macara, I.G., 2012. The Scribble polarity protein stabilizes E-cadherin/p120-catenin binding and blocks retrieval of E-cadherin to the Golgi. *PloS one*, 7, p.e51130.

Longworth, M.S. & Laimins, L.A., 2004. Pathogenesis of Human Papillomaviruses in Differentiating Epithelia. *Society*, 68, pp.362–372.

Longworth, M.S., Wilson, R. & Laimins, L.A., 2005. HPV31 E7 facilitates replication by activating E2F2 transcription through its interaction with HDACs. *the The European Molecular Biology Organization Journal*, 24, pp.1821–1830.

Ma, X.M. & Blenis, J., 2009. Molecular mechanisms of mTOR-mediated translational control. *Nature Reviews Molecular Cell Biology*, 10, pp.307–318.

Makokha, G.N. *et al.*, 2013. Human T-cell leukemia virus type 1 Tax protein interacts with and mislocalizes the PDZ domain protein MAGI-1. *Cancer Sci.*

Malanchi, I. *et al.*, 2004. Human papillomavirus type 16 E6 promotes retinoblastoma protein phosphorylation and cell cycle progression. *Journal of Virology*, 78, pp.13769–13778.

Manning, A.L. & Dyson, N.J., 2011. pRB, a tumor suppressor with a stabilizing presence. *Trends in Cell Biology*, 21, pp.433–441.

Mantovani, F., Massimi, P. & Banks, L., 2001. Proteasome-mediated regulation of the hDlg tumour suppressor protein. *Journal of Cell Science*, 114, pp.4285–4292.

Martin, K.A. & Blenis, J., 2002. Coordinate regulation of translation by the PI 3-kinase and mTOR pathways. *Advances in Cancer Research*, 86, pp.1–39.

Martin-Belmonte, F. & Perez-Moreno, M., 2011. Epithelial cell polarity, stem cells and cancer. *Nature Reviews Cancer*, 12, pp.23–38.

Massimi, P. *et al.*, 2012. Differential regulation of cell-cell contact, invasion and anoikis by hScrib and hDlg in keratinocytes. *PloS one*, 7, p.e40279.

Massimi, P. *et al.*, 2008. HPV E6 degradation of p53 and PDZ containing substrates in an E6AP null background. *Oncogene*, 27, pp.1800–1804.

Massimi, P. *et al.*, 2004. HPV E6 specifically targets different cellular pools of its PDZ domain-containing tumour suppressor substrates for proteasome-mediated degradation. *Oncogene*, 23, pp.8033–8039.

Massimi, P. *et al.*, 2006. Phosphorylation of the discs large tumour suppressor protein controls its membrane localisation and enhances its susceptibility to HPV E6-induced degradation. *Oncogene*, 25, pp.4276–4285.

Matlashewski, G. *et al.*, 1987. Human papillomavirus type 16 DNA cooperates with activated ras in transforming primary cells. *the The European Molecular Biology Organization Journal*, 6, pp.1741–1746.

Matsuda, I. & Mishina, M., 2000. Identification of a juxtamembrane segment of the glutamate receptor delta2 subunit required for the plasma membrane localization. *Biochemical and Biophysical Research Communications*, 275, pp.565–571.

Matsukura, T., Koi, S. & Sugase, M., 1989. Both episomal and integrated forms of human papillomavirus type 16 are involved in invasive cervical cancers. *Virology*, 172, pp.63–72.

Matsumine, A. *et al.*, 1996. Binding of APC to the human homolog of the Drosophila discs large tumor suppressor protein. *Science*, 272, pp.1020–1023.

Matthews, K. *et al.*, 2003. Depletion of Langerhans Cells in Human Papillomavirus Type 16-Infected Skin Is Associated with E6-Mediated Down Regulation of E-Cadherin. *Journal of Virology*, 77, pp.8378–8385.

Mccaffrey, L.M. & Macara, I.G., 2011. Epithelial organization , cell polarity and tumorigenesis. *Trends in Cell Biology*, 21, pp.727–735.

McIntosh, P.B. *et al.*, 2008. Structural Analysis Reveals an Amyloid Form of the Human Papillomavirus Type 16 E1∧E4 Protein and Provides a Molecular Basis for Its Accumulation. *Journal of Virology*, 82, pp.8196–8203.

McPhillips, M.G. *et al.*, 2006. Brd4 Is Required for E2-Mediated Transcriptional Activation but Not Genome Partitioning of All Papillomaviruses. *Journal of Virology*, 80, pp.9530–9543.

Meisels, A. & Fortin, R., 1976. Condylomatous lesions of the cervix and vagina. I. Cytologic patterns. *Acta Cytologica*, 20, pp.505–509.

Menges, C.W. *et al.*, 2006. Human papillomavirus type 16 E7 up-regulates AKT activity through the retinoblastoma protein. *Cancer Research*, 66, pp.5555–5559.

Middleton, K. *et al.*, 2003. Organization of human papillomavirus productive cycle during neoplastic progression provides a basis for selection of diagnostic markers. *J Virol*, 77, pp.10186–10201.

Mietz, J.A. *et al.*, 1992. The transcriptional transactivation function of wild-type p53 is inhibited by SV40 large T-antigen and by HPV-16 E6 oncoprotein. *The EMBO Journal*, 11, pp.5013–5020.

Mino, A. *et al.*, 2000. Membrane-associated guanylate kinase with inverted orientation (MAGI)-1/brain angiogenesis inhibitor 1-associated protein (BAP1) as a scaffolding molecule for Rap small G protein GDP/GTP exchange protein at tight junctions. *Genes to cells devoted to molecular cellular mechanisms*, 5, pp.1009–1016.

Miyoshi, Y. *et al.*, 1992. Germ-line mutations of the APC gene in 53 familial adenomatous polyposis patients. *Proceedings of the National Academy of Sciences of the United States of America*, 96, pp.4452–4456.

Monnier-Benoit, S. *et al.*, 2006. Immunohistochemical analysis of CD4+ and CD8+ T-cell subsets in high risk human papillomavirus-associated pre-malignant and malignant lesions of the uterine cervix. *Gynecol Oncol*, 102, pp.22–31.

Moore, P.S. & Chang, Y., 2010. Why do viruses cause cancer? Highlights of the first century of human tumour virology. *Nature Reviews Cancer*, 10, pp.878–889.

Morais Cabral, J.H. *et al.*, 1996. Crystal structure of a PDZ domain. *Nature*, 382, p.771.

Munger, K. *et al.*, 1989. Complex formation of c-myc papillomavirus E7 proteins with the retinoblastoma tumor suppressor gene product. *EMBO Journal*, 8, pp.4099–4105.

Murata, M. *et al.*, 2005. Tight junction protein MAGI-1 is up-regulated by transfection with connexin 32 in an immortalized mouse hepatic cell line: cDNA microarray analysis. *Cell and Tissue Research*, 319, pp.341–347.

Muroyama, A. & Lechler, T., 2012. Polarity and stratification of the epidermis. *Seminars in cell developmental biology*, 23, pp.890–6.

Murphy, C.G. & Morris, P.G., 2012. Recent advances in novel targeted therapies for HER2-positive breast cancer. *Anticancer drugs*, 23, pp.765–776.

Murray, D. *et al.*, 2008. NET1-mediated RhoA activation facilitates lysophosphatidic acid-induced cell migration and invasion in gastric cancer. *British Journal of Cancer*, 99, pp.1322–1329.

Nagafuchi, A., 2001. Molecular architecture of adherens junctions. *Current Opinion in Cell Biology*, 13, pp.600–603.

Nagasaka, K. *et al.*, 2010. The cell polarity regulator hScrib controls ERK activation through a KIM site-dependent interaction. *Oncogene*, 29, pp.5311–5321.

Nakagawa, S. *et al.*, 2004. Analysis of the expression and localisation of a LAP protein, human scribble, in the normal and neoplastic epithelium of uterine cervix. *British Journal of Cancer*, 90, pp.194–199.

Nakagawa, S. & Huibregtse, J.M., 2000. Human scribble (Vartul) is targeted for ubiquitin-mediated degradation by the high-risk papillomavirus E6 proteins and the E6AP ubiquitin-protein ligase. *Molecular and cellular biology*, 20, pp.8244–53.

Nakahara, T. *et al.*, 2005. Human papillomavirus type 16 E1circumflexE4 contributes to multiple facets of the papillomavirus life cycle. *Journal of Virology*, 79, pp.13150–13165.

Narayan, N., Subbaiah, V.K. & Banks, L., 2009. The high-risk HPV E6 oncoprotein preferentially targets phosphorylated nuclear forms of hDlg. *Virology*, 387, pp.1–4.

Navarro, C. *et al.*, 2005. Junctional recruitment of mammalian Scribble relies on E-cadherin engagement. *Oncogene*, 24, pp.4330–4339.

Nees, M. *et al.*, 2001. Papillomavirus type 16 oncogenes downregulate expression of interferon-responsive genes and upregulate proliferation-associated and NF-kappaB-responsive genes in cervical keratinocytes. *Journal of virology*, 75, pp.4283–4296.

Nguyen, J.T. *et al.*, 1998. Exploiting the basis of proline recognition by SH3 and WW domains: design of N-substituted inhibitors. *Science*, 282, pp.2088–2092.

Nguyen, M. *et al.*, 2002. A Mutant of Human Papillomavirus Type 16 E6 Deficient in Binding α-Helix Partners Displays Reduced Oncogenic Potential In Vivo. *Journal of Virology*, 76, pp.13039–13048.

Nguyen, M.L. *et al.*, 2003. The PDZ Ligand Domain of the Human Papillomavirus Type 16 E6 Protein Is Required for E6's Induction of Epithelial Hyperplasia In Vivo. *Journal of Virology*, 77, pp.6957–6964.

Nicholls, P.K. *et al.*, 2001. Regression of canine oral papillomas is associated with infiltration of CD4+ and CD8+ lymphocytes. *Virology*, 283, pp.31–39.

Nicolaides, L. *et al.*, 2011. Stabilization of HPV16 E6 protein by PDZ proteins, and potential implications for genome maintenance. *Virology*, 414, pp.137–145.

Nie, M. *et al.*, 2009. The Y-box factor ZONAB/DbpA associates with GEF-H1/Lfc and mediates Rho-stimulated transcription. *EMBO Reports*, 10, pp.1125–1131.

Niethammer, M. *et al.*, 1998. CRIPT, a novel postsynaptic protein that binds to the third PDZ domain of PSD-95/SAP90. *Neuron*, 20, pp.693–707.

Nieto, M.A., 2002. The snail superfamily of zinc-finger transcription factors. *Nature Reviews Molecular Cell Biology*, 3, pp.155–166.

Nourry, C., Grant, S.G.N. & Borg, J.-P., 2003. PDZ domain proteins: plug and play! *Sciences STKE signal transduction knowledge environment*, 2003, p.RE7.

Nunbhakdi-Craig, V. *et al.*, 2002. Protein phosphatase 2A associates with and regulates atypical PKC and the epithelial tight junction complex. *The Journal of Cell Biology*, 158, pp.967–978.

Oberhammer, F.A. *et al.*, 1994. Chromatin condensation during apoptosis is accompanied by degradation of lamin A+B, without enhanced activation of cdc2 kinase. *The Journal of Cell Biology*, 126, pp.827–837.

Ohashi, M. *et al.*, 2004. Human T-cell leukemia virus type 1 Tax oncoprotein induces and interacts with a multi-PDZ domain protein, MAGI-3. *Virology*, 320, pp.52–62.

Okajima, M. *et al.*, 2008. Human T-cell leukemia virus type 1 Tax induces an aberrant clustering of the tumor suppressor Scribble through the PDZ domain-binding motif dependent and independent interaction. *Virus Genes*, 37, pp.231–240.

Okamoto, K. *et al.*, 2006. Human T-cell leukemia virus type-I oncoprotein Tax inhibits Fas-mediated apoptosis by inducing cellular FLIP through activation of NF-kappaB. *Genes to cells devoted to molecular cellular mechanisms*, 11, pp.177–191.

Ozdamar, B. *et al.*, 2005. Regulation of the polarity protein Par6 by TGFß peceptors controls epithelial cell plasticity. *Science*, 307, pp.1603–1609.

Pan, D., 2010. The hippo signaling pathway in development and cancer. *Developmental Cell*, 19, pp.491–505.

Parada, L.F. *et al.*, 1982. Human EJ bladder carcinoma oncogene is homologue of Harvey sarcoma virus ras gene. *Nature*, 297, pp.474–478.

Paramio, J.M. *et al.*, 1998. Differential expression and functionally co-operative roles for the retinoblastoma family of proteins in epidermal differentiation. *Oncogene*, 17, pp.949–957.

Parish, J.L. *et al.*, 2006. E2 Proteins from High- and Low-Risk Human Papillomavirus Types Differ in Their Ability To Bind p53 and Induce Apoptotic Cell Death. *Journal of Virology*, 80, pp.4580–4590.

Parkin, D.M. & Bray, F., 2006. Chapter 2: The burden of HPV-related cancers. *Vaccine*, 24 Suppl 3, pp.S3/11–25.

Parreño, M. *et al.*, 2001. E1A modulates phosphorylation of p130 and p107 by differentially regulating the activity of G1/S cyclin/CDK complexes. *Oncogene*, 20, pp.4793–4806.

Patel, D. *et al.*, 1999. The E6 protein of human papillomavirus type 16 binds to and inhibits co-activation by CBP and p300. *the The European Molecular Biology Organization Journal*, 18, pp.5061–72.

Pedemonte, S. *et al.*, 1998. Novel germline APC variants in patients with multiple adenomas. *Genes chromosomes cancer*, 22, pp.257–267.

Peh, W.L. *et al.*, 2004. The Viral E4 Protein Is Required for the Completion of the Cottontail Rabbit Papillomavirus Productive Cycle In Vivo. *Journal of Virology*, 78, pp.2142–2151.

Peinado, H., Olmeda, D. & Cano, A., 2007. Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype? *Nat Rev Cancer*, 7, pp.415–428.

Peng, X. *et al.*, 2012. Overexpression of cystic fibrosis transmembrane conductance regulator (CFTR) is associated with human cervical cancer malignancy, progression and prognosis. *Gynecologic Oncology*, 125, pp.470–6.

Perea, S.E., Massimi, P. & Banks, L., 2000. Human papillomavirus type 16 E7 impairs the activation of the interferon regulatory factor-1. *International Journal of Molecular Medicine*, 5, pp.661–666.

Pestova, T. V *et al.*, 2001. Molecular mechanisms of translation initiation in eukaryotes. *Proceedings of the National Academy of Sciences of the United States of America*, 98, pp.7029–7036.

Pett, M. & Coleman, N., 2007. Integration of high-risk human papillomavirus: a key event in cervical carcinogenesis? *The Journal of pathology*, 212, pp.356–367.

Pim, D. *et al.*, 2005. Activation of the protein kinase B pathway by the HPV-16 E7 oncoprotein occurs through a mechanism involving interaction with PP2A. *Oncogene*, 24, pp.7830–7838.

Pim, D. *et al.*, 2000. HPV E6 targeted degradation of the discs large protein: evidence for the involvement of a novel ubiquitin ligase. *Oncogene*, 19, pp.719–725.

Pim, D. *et al.*, 1994. Mutational analysis of HPV-18 E6 identifies domains required for p53 degradation in vitro, abolition of p53 transactivation in vivo and immortalisation of primary BMK cells. *Oncogene*, 9, pp.1869–1876.

Pim, D., Collins, M. & Banks, L., 1992. Human papillomavirus type 16 E5 gene stimulates the transforming activity of the epidermal growth factor receptor. *Oncogene*, 7, pp.27–32.

Pirami, L., Giachè, V. & Becciolini, A., 1997. Analysis of HPV16, 18, 31, and 35 DNA in pre-invasive and invasive lesions of the uterine cervix. *Journal of Clinical Pathology*, 50, pp.600–604.

Plant, P.J. *et al.*, 2003. A polarity complex of mPar-6 and atypical PKC binds, phosphorylates and regulates mammalian Lgl. *Nature Cell Biology*, 5, pp.301–308.

Pullen, N. & Thomas, G., 1997. The modular phosphorylation and activation of p70(s6k). *FEBS Letters*, 410, pp.78–82.

Pyeon, D. *et al.*, 2009. Establishment of Human Papillomavirus Infection Requires Cell Cycle Progression. *PLoS Pathogens*, 5, p.9.

Qin, Y. *et al.*, 2005. The mammalian Scribble polarity protein regulates epithelial cell adhesion and migration through E-cadherin. *The Journal of Cell Biology*, 171, pp.1061–1071.

Rachow, S. *et al.*, 2013. Occludin is involved in adhesion, apoptosis, differentiation and ca(2+)-homeostasis of human keratinocytes: implications for tumorigenesis. *PloS one*, 8, p.e55116.

Ray, P.S., Grover, R. & Das, S., 2006. Two internal ribosome entry sites mediate the translation of p53 isoforms. *EMBO Reports*, 7, pp.404–410.

Reinhard, C. *et al.*, 1994. Nuclear localization of p85s6k: functional requirement for entry into S phase. *the The European Molecular Biology Organization Journal*, 13, pp.1557–1565.

Reynaud, C., Fabre, S. & Jalinot, P., 2000. The PDZ protein TIP-1 interacts with the Rho effector rhotekin and is involved in Rho signaling to the serum response element. *The Journal of Biological Chemistry*, 275, pp.33962–33968.

Reynolds, A.B. *et al.*, 1994. Identification of a new catenin: the tyrosine kinase substrate p120cas associates with E-cadherin complexes. *Molecular and Cellular Biology*, 14, pp.8333–8342.

Riley, R.R. *et al.*, 2003. Dissection of human papillomavirus E6 and E7 function in transgenic mouse models of cervical carcinogenesis. *Cancer Research*, 63, pp.4862–4871.

Roegiers, F. & Jan, Y.N., 2004. Cell adhesion in regulation of asymmetric stem cell division. *Current Opinion in Cell Biology*, 22, pp.195–205.

Roh, M.H. *et al.*, 2002. The carboxyl terminus of zona occludens-3 binds and recruits a mammalian homologue of discs lost to tight junctions. *The Journal of Biological Chemistry*, 277, pp.27501–27509.

Roh, M.H. & Margolis, B., 2003. Composition and function of PDZ protein complexes during cell polarization. *Am J Physiol Renal Physiol*, 285, pp.F377–87.

Rosner, M. & Hengstschläger, M., 2011a. Nucleocytoplasmic localization of p70 S6K1, but not of its isoforms p85 and p31, is regulated by TSC2/mTOR. *Oncogene*, 30, pp.4509–22.

Rosner, M. & Hengstschläger, M., 2011b. Nucleocytoplasmic localization of p70 S6K1, but not of its isoforms p85 and p31, is regulated by TSC2/mTOR. *Oncogene*, 30, pp.4509–22.

Ross, S.R., Flint, S.J. & Levine, A.J., 1980. Identification of the adenovirus early proteins and their genomic map positions. *Virology*, 100, pp.419-32.

Ross, S.R. *et al.*, 1980. Early viral proteins in HeLa cells infected with adenovirus type 5 host range mutants. *Virology*, 103, pp.475-92.

Rous, P. & Beard, J.W., 1934. A VIRUS-INDUCED MAMMALIAN GROWTH WITH THE CHARACTERS OF A TUMOR (THE SHOPE RABBIT PAPILLOMA) : I. THE GROWTH ON IMPLANTATION WITHIN FAVORABLE HOSTS. *The Journal of Experimental Medicine*, 60, pp.701–722.

Rous P., 1910. A transmissable avian neoplasm. *The Journal of Experimental Medicine*,12, pp. 696–705.

Rous P., 1911. A sarcoma of the fowl transmissible by an agent separable from the tumor cells. *The Journal of Experimental Medicine*, 13, pp. 397–9.

Rousset, R. *et al.*, 1998. The C-terminus of the HTLV-1 Tax oncoprotein mediates interaction with the PDZ domain of cellular proteins. *Oncogene*, 16, pp.643–654.

Rowe, W.P., *et al.*, 1953. Isolation of a cytopathogenic agent from human adenoids undergoing spontaneous degeneration in tissue culture. *Proceedings of the Society for Experimental Biology and Medicine*, 84, pp. 570–573.

Saitou, M. *et al.*, 2000. Complex Phenotype of Mice Lacking Occludin, a Component of Tight Junction Strands. *Molecular Biology of the Cell*, 11, pp.4131–4142.

Sakurai, A. *et al.*, 2006. MAGI-1 is required for Rap1 activation upon cell-cell contact and for enhancement of vascular endothelial cadherin-mediated cell adhesion. *Molecular Biology of the Cell*, 17, pp.966–976.

Salahshor, S. *et al.*, 2008. Frequent accumulation of nuclear E-cadherin and alterations in the Wnt signaling pathway in esophageal squamous cell carcinomas. *Modern pathology an official journal of the United States and Canadian Academy of Pathology Inc*, 21, pp.271–281.

Salic, A. & Mitchison, T.J., 2008. A chemical method for fast and sensitive detection of DNA synthesis in vivo. *Proceedings of the National Academy of Sciences of the United States of America*, 105, pp.2415–2420.

Salmena, L., Carracedo, A. & Pandolfi, P.P., 2008. Tenets of PTEN tumor suppression. *Cell*, 133, pp.403–14.

Sambrook, J., *et al.*, 1968. The integrated state of viral DNA in SV40-transformed cells. *Proceedings of the National Academy of Sciences of the United States of America*, 60, 1288-1295.

Sambrook, J. *et al.*, 1980. Integration of viral DNA sequences in cells transformed by adenovirus 2 or SV40. *Proceedings of the Royal Society of London Series B Containing papers of a Biological character Royal Society Great Britain*, 210, pp.423–435.

Samuels-Lev, Y. *et al.*, 2001. ASPP proteins specifically stimulate the apoptotic function of p53. *Molecular Cell*, 8, pp.781–794.

Saras, J. *et al.*, 1997. Characterization of the interactions between PDZ domains of the protein-tyrosine phosphatase PTPL1 and the carboxyl-terminal tail of Fas. *The Journal of Biological Chemistry*, 272, pp.20979–20981.

Sarbassov, D.D. *et al.*, 2005. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science New York NY*, 307, pp.1098–101.

Sarnow, P. *et al.*, 1982. Adenovirus E1b-58kd tumor antigen and SV40 large tumor antigen are physically associated with the same 54 kd cellular protein in transformed cells. *Cell*, 28, pp.387–394.

Sarver, N. *et al.*, 1984. Localization and analysis of bovine papillomavirus type 1 transforming functions. *Journal of Virology*, 52, pp.377–388.

Scheffner, M. *et al.*, 1990. The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. *Cell*, 63, pp.1129–1136.

Scheffner, M. *et al.*, 1993. The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53. *Cell*, 75, pp.495–505.

Scheid, M.P. *et al.*, 2002. Multiple Phosphoinositide 3-Kinase-Dependent Steps in Activation of Protein Kinase B Multiple Phosphoinositide 3-Kinase-Dependent Steps in Activation of Protein Kinase B. *Society*, 22, pp.6247–6260.

Schimanski, C.C. *et al.*, 2005. Reduced expression of Hugl-1, the human homologue of Drosophila tumour suppressor gene lgl, contributes to progression of colorectal cancer. *Oncogene*, 24, pp.3100–3109.

Schlegel, R. *et al.*, 1988. Quantitative keratinocyte assay detects two biological activities of human papillomavirus DNA and identifies viral types associated with cervical carcinoma. *the The European Molecular Biology Organization Journal*, 7, pp.3181–3187.

Schneeberger, E.E. & Lynch, R.D., 2004. The tight junction: a multifunctional complex. *American journal of physiology Cell physiology*, 286, pp.C1213–C1228.

Schneider-Gädicke, A. & Schwarz, E., 1986. Different human cervical carcinoma cell lines show similar transcription patterns of human papillomavirus type 18 early genes. *the The European Molecular Biology Organization Journal*, 5, pp.2285–2292.

Schwarz, E. *et al.*, 1983. DNA sequence and genome organization of genital human papillomavirus type 6b. *the The European Molecular Biology Organization Journal*, 2, pp.2341–2348.

Schwarz, E. *et al.*, 1985. Structure and transcription of human papillomavirus sequences in cervical carcinoma cells. *Nature*, 314, pp.111–114.

Sedman, T., Sedman, J. & Stenlund, A., 1997. Binding of the E1 and E2 proteins to the origin of replication of bovine papillomavirus. *Journal of Virology*, 71, pp.2887–2896.

Sekaric, P. *et al.*, 2007. hAda3 regulates p14ARF-induced p53 acetylation and senescence. *Oncogene*, 26, pp.6261–6268.

Serra, S. *et al.*, 2007. Nuclear expression of E-cadherin in solid pseudopapillary tumors of the pancreas. *JOP Journal of the pancreas*, 8, pp.296–303.

Serrano, M., Hannon, G.J. & Beach, D., 1993. A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. *Nature*, 366, pp.704–707.

Shai, A. *et al.*, 2007. The Human Papillomavirus E6 Oncogene Dysregulates the Cell Cycle and Contributes to Cervical Carcinogenesis through Two Independent Activities 10.1158/0008-5472.CAN-06-3344. *Cancer Research*, 67, pp.1626–1635.

Shai, A., Pitot, H.C. & Lambert, P.F., 2010. E6-associated protein is required for human papillomavirus type 16 E6 to cause cervical cancer in mice. *Cancer Research*, 70, pp.5064–5073.

Shamanin, V.A., Sekaric, P. & Androphy, E.J., 2008. hAda3 degradation by papillomavirus type 16 E6 correlates with abrogation of the p14ARF-p53 pathway and efficient immortalization of human mammary epithelial cells. *Journal of Virology*, 82, pp.3912–3920.

Shein, H.M., 1967. Transformation of astrocytes and destruction of spongioblasts induced by a simian tumor virus (SV40) in cultures of human fetal neuroglia. *Journal of Neuropathology and Experimental Neurology*, 26, pp. 60-76.

Sherr, C.J. & Weber, J.D., 2000. The ARF/p53 pathway. *Current opinion in genetics development*, 10, pp.94–9.

Shisler, J. *et al.*, 1997. The adenovirus E3-10.4K/14.5K complex mediates loss of cell surface Fas (CD95) and resistance to Fas-induced apoptosis. *Journal of Virology*, 71, pp.8299–8306.

Shope, R.E. & Hurst, E.W., 1933. INFECTIOUS PAPILLOMATOSIS OF RABBITS : WITH A NOTE ON THE HISTOPATHOLOGY. *The Journal of Experimental Medicine*, 58, pp.607–624.

Simonson, S.J.S., Difilippantonio, M.J. & Lambert, P.F., 2005. Two distinct activities contribute to human papillomavirus 16 E6's oncogenic potential. *Cancer Research*, 65, pp.8266–8273.

Van Den Elsen, P.J., Houweling, A. & Van Der Eb, A.J., 1983. Morphological transformation of human adenoviruses is determined to a large extent by gene products of region E1a. *Virology*, 131, pp.242–246.

Van Ham, M. & Hendriks, W., 2003. PDZ domains-glue and guide. *Molecular Biology Reports*, 30, pp.69–82.

Seif, R. & Martin, R.G., 1979. Growth state of the cell early after infection with simian virus 40 determines whether the maintenance of transformation will be A-gene dependent or independent. *Journal of Virology*, 31, pp.350–359.

Shuda, M., *et al.*, 2008. T antigen mutations are a human tumor-specific signature for Merkel cell polyomavirus. *Proceedings of the National Academy of Sciences of the United States of America*, 105, pp. 16272-16277.

Shuda, M., *et al.*, 2009. Human Merkel cell polyomavirus infection I. MCV T antigen expression in Merkel cell carcinoma, lymphoid tissues and lymphoid tumors. *Interbational Journal of Cancer*, 125, 1243-1249

Skiadopoulos, M.H. & McBride, A.A., 1998. Bovine papillomavirus type 1 genomes and the E2 transactivator protein are closely associated with mitotic chromatin. *Journal of Virology*, 72, pp.2079–2088.

Sleigh, M.J. *et al.*, 1978. Mutants of SV40 with an altered small t protein are reduced in their ability to transform cells. *Cell*, 14, pp.79–88.

Smits, V.A. & Medema, R.H., 2001. Checking out the G(2)/M transition. *Biochimica et Biophysica Acta*, 1519, pp.1–12.

Smotkin, D. & Wettstein, F.O., 1986. Transcription of human papillomavirus type 16 early genes in a cervical cancer and a cancer-derived cell line and identification of the E7 protein. *Proceedings of the National Academy of Sciences of the United States of America*, 83, pp.4680–4684.

Song, S. *et al.*, 2000. Human papillomavirus types 16 E6 and E7 contribute differently to carcinogenesis. *Virology*, 267, pp.141–150.

Song, S., Pitot, H.C. & Lambert, P.F., 1999. The human papillomavirus type 16 E6 gene alone is sufficient to induce carcinomas in transgenic animals. *Journal of Virology*, 73, pp.5887–5893.

Songyang, Z. *et al.*, 1997. Recognition of unique carboxyl-terminal motifs by distinct PDZ domains. *Science*, 275, pp.73–77.

Sottocornola, R. *et al.*, 2010. ASPP2 Binds Par-3 and Controls the Polarity and Proliferation of Neural Progenitors during CNS Development. *Developmental Cell*, 19, pp.126–137.

Sourisseau, T. *et al.*, 2006. Regulation of PCNA and Cyclin D1 Expression and Epithelial Morphogenesis by the ZO-1-Regulated Transcription Factor ZONAB/DbpA. *Molecular and Cellular Biology*, 26, pp.2387–2398.

Spangle, J.M., Ghosh-Choudhury, N. & Munger, K., 2012. Activation of cap-dependent translation by mucosal human papillomavirus E6 proteins is dependent on the integrity of the LXXLL binding motif. *Journal of virology*, 86, pp.7466–7472.

Spangle, J.M. & Münger, K., 2010. The Human Papillomavirus Type 16 E6 Oncoprotein Activates mTORC1 Signaling and Increases Protein Synthesis. *Journal of Virology*, 84, pp.9398–9407.

Spangle, J.M. & Munger, K., 2013. The HPV16 E6 Oncoprotein Causes Prolonged Receptor Protein Tyrosine Kinase Signaling and Enhances Internalization of Phosphorylated Receptor Species. *PLoS pathogens*, 9, p.e1003237.

Spanos, W.C., Geiger, J., *et al.*, 2008. Deletion of the PDZ motif of HPV16 E6 preventing immortalization and anchorage-independent growth in human tonsil epithelial cells. *Head neck*, 30, pp.139–147.

Spanos, W.C., Hoover, A., *et al.*, 2008. The PDZ Binding Motif of Human Papillomavirus Type 16 E6 Induces PTPN13 Loss, Which Allows Anchorage-Independent Growth and Synergizes with Ras for Invasive Growth. *Journal of Virology*, 82, pp.2493–2500.

Sprecher, E. & Becker, Y., 1993a. Role of Langerhans cells and other dendritic cells in disease states. *In vivo Athens Greece*, 7, pp.217–227.

Sprecher, E. & Becker, Y., 1993b. Role of Langerhans cells and other dendritic cells in viral diseases. *Archives of Virology*, 132, pp.1–28.

St Johnston, D. & Ahringer, J., 2010. Cell polarity in eggs and epithelia: parallels and diversity. *Cell*, 141, pp.757–774.

Stacey, S.N. *et al.*, 1995. Translation of the human papillomavirus type 16 E7 oncoprotein from bicistronic mRNA is independent of splicing events within the E6 open reading frame. *Journal of Virology*, 69, pp.7023–7031.

Stacey, S.N. *et al.*, 2000. Leaky scanning is the predominant mechanism for translation of human papillomavirus type 16 E7 oncoprotein from E6/E7 bicistronic mRNA. *Journal of Virology*, 74, pp.7284–7297.

Stanley, M.A., 2012. Epithelial cell responses to infection with human papillomavirus. *Clinical Microbiology Reviews*, 25, pp.215–22.

Steger, G. & Corbach, S., 1997. Dose-Dependent Regulation of the Early Promoter of Human Papillomavirus Type 18 by the Viral E2 Protein. *Microbiology*, 71, pp.50–58.

Sterling, J. *et al.*, 1990. Production of human papillomavirus type 16 virions in a keratinocyte cell line. *Journal of Virology*, 64, pp.6305–6307.

Stevenson, B.R. *et al.*, 1986. Identification of ZO-1: a high molecular weight polypeptide associated with the tight junction (zonula occludens) in a variety of epithelia. *The Journal of Cell Biology*, 103, pp.755–766.

Stewart, D. *et al.*, 2004. Ubiquitination and proteasome degradation of the E6 proteins of human papillomavirus types 11 and 18. *Journal of General Virology*, 85, pp.1419–1426.

Stewart, D., Ghosh, A. & Matlashewski, G., 2005. Involvement of nuclear export in human papillomavirus type 18 E6-mediated ubiquitination and degradation of p53. *Journal of Virology*, 79, pp.8773–8783.

Stewart, H.L., 1953, Pulmonary tumors in animals with particular reference to mice. *Acta-Unio Internationalis Contra Cancrum*, 9, pp 512-528.

Storrs, C.H. & Silverstein, S.J., 2007. PATJ a Tight Junction-Associated PDZ Protein is a Novel Degradation Target of High-Risk HPV E6 and the Alternatively Spliced Isoform 18 E6. *Journal of Virology*, pp.JVI.02545–06.

Stott, F.J. *et al.*, 1998. The alternative product from the human CDKN2A locus, p14(ARF), participates in a regulatory feedback loop with p53 and MDM2. *the The European Molecular Biology Organization Journal*, 17, pp.5001–5014.

Strati, K. & Lambert, P.F., 2007. Role of Rb-dependent and Rb-independent functions of papillomavirus E7 oncogene in head and neck cancer. *Cancer Research*, 67, pp.11585–11593.

Stricker, N.L. *et al.*, 1997. PDZ domain of neuronal nitric oxide synthase recognizes novel C-terminal peptide sequences. *Nature Biotechnology*, 15, pp.336–342.

Stubenrauch, F. & Laimins, L.A., 1999. Human papillomavirus life cycle: active and latent phases. *Semin Cancer Biol*, 9, pp.379–386.

Stubenrauch, F., Lim, H.B. & Laimins, L.A., 1998. Differential Requirements for Conserved E2 Binding Sites in the Life Cycle of Oncogenic Human Papillomavirus Type 31. *Journal of Virology*, 72, pp.1071–1077.

Subbaiah, V.K. *et al.*, 2011. PDZ domains: the building blocks regulating tumorigenesis. *The Biochemical journal*, 439, pp.195–205.

Sukumar, S. *et al.*, 1984. A transforming ras gene in tumorigenic guinea pig cell lines initiated by diverse chemical carcinogens. *Science*, 223, pp.1197–1199.

Suzuki, A., 2002. aPKC kinase activity is required for the asymmetric differentiation of the premature junctional complex during epithelial cell polarization. *Journal of Cell Science*, 115, pp.3565–3573.

Suzuki-Takahashi, I. *et al.*, 1995. The interactions of E2F with pRB and with p107 are regulated via the phosphorylation of pRB and p107 by a cyclin-dependent kinase. *Oncogene*, 10, pp.1691–1698.

Sweeney, C. *et al.*, 2001. Growth factor-specific signaling pathway stimulation and gene expression mediated by ErbB receptors. *The Journal of Biological Chemistry*, 276, pp.22685–22698.

Sweet, B.H., Hilleman, M.R., 1960. The vacuolating virus, S.V. 40. *Proceedings of the Society for Experimental Biology and Medicine*, 105, pp. 420-427.

Syverton, J.T., Berry, G.P., 1935. Carcinoma in cottontail rabbit following spontaneous virus papilloma (Shope). *Proc. Sac. Exp. Biol. Med*, 33, p. 399.

Talis, A.L., Huibregtse, J.M. & Howley, P.M., 1998. The role of E6AP in the regulation of p53 protein levels in human papillomavirus (HPV)-positive and HPV-negative cells. *The Journal of Biological Chemistry*, 273, pp.6439–6445.

Tamura, A. *et al.*, 2008. Megaintestine in claudin-15-deficient mice. *Gastroenterology*, 134, pp.523–534.

Tan, T.M. *et al.*, 1994. Mechanism of translation of the bicistronic mRNA encoding human papillomavirus type 16 E6-E7 genes. *The Journal of general virology*, 75 (Pt 10, pp.2663–2670.

Tang, A. *et al.*, 1993. Adhesion of epidermal Langerhans cells to keratinocytes mediated by E-cadherin. *Nature*, 361, pp.82–85.

Tang, S. *et al.*, 2006. The E7 oncoprotein is translated from spliced E6*I transcripts in high-risk human papillomavirus type 16- or type 18-positive cervical cancer cell lines via translation reinitiation. *Journal of Virology*, 80, pp.4249–4263.

Tang, V.W., 2006. Proteomic and bioinformatic analysis of epithelial tight junction reveals an unexpected cluster of synaptic molecules. *Biology Direct*, 1, p.37.

Taylor, R.C., Cullen, S.P. & Martin, S.J., 2008. Apoptosis: controlled demolition at the cellular level. *Nature Reviews Molecular Cell Biology*, 9, pp.231–241.

Thiery, J.P. *et al.*, 2009. Epithelial-mesenchymal transitions in development and disease. *Cell*, 139, pp.871–90.

Thomas, M. *et al.*, 1996. Induction of apoptosis by p53 is independent of its oligomeric state and can be abolished by HPV-18 E6 through ubiquitin mediated degradation. *Oncogene*, 13, pp.265–273.

Thomas, U. *et al.*, 1997. Functional expression of rat synapse-associated proteins SAP97 and SAP102 in Drosophila dlg-1 mutants: effects on tumor suppression and synaptic bouton structure. *Mechanisms of Development*, 62, pp.161–174.

Thomas, D.L. *et al.*, 1999. Early region 1 transforming functions are dispensable for mammary tumorigenesis by human adenovirus type 9. *J Virol*, 73, pp.3071–3079.

Thomas, M. *et al.*, 2008. Analysis of specificity determinants in the interactions of different HPV E6 proteins with their PDZ domain-containing substrates. *Virology*, 376, pp.371–378.

Thomas, M. *et al.*, 2001. HPV E6 and MAGUK protein interactions: determination of the molecular basis for specific protein recognition and degradation. *Oncogene*, 20, pp.5431–5439.

Thomas, M. *et al.*, 2008. Human papillomaviruses, cervical cancer and cell polarity. *Oncogene*, 27, pp.7018–7030.

Thomas, M. *et al.*, 2002. Oncogenic human papillomavirus E6 proteins target the MAGI-2 and MAGI-3 proteins for degradation. *Oncogene*, 21, pp.5088–5096.

Thomas, M. *et al.*, 2005. The hScrib/Dlg apico-basal control complex is differentially targeted by HPV-16 and HPV-18 E6 proteins. *Oncogene*, 24, pp.6222–6230.

Thomas, M.C. & Chiang, C.-M., 2005. E6 oncoprotein represses p53-dependent gene activation via inhibition of protein acetylation independently of inducing p53 degradation. *Molecular Cell*, 17, pp.251–64.

Tian, Q. *et al.*, 1991. A polyadenylate binding protein localized to the granules of cytolytic lymphocytes induces DNA fragmentation in target cells. *Cell*, 67, pp.629–639.

Toker, A. & Newton, A.C., 2000. Akt/protein kinase B is regulated by autophosphorylation at the hypothetical PDK-2 site. *The Journal of Biological Chemistry*, 275, pp.8271–8274.

Tollefson, A.E. *et al.*, 1998. Forced degradation of Fas inhibits apoptosis in adenovirus-infected cells. *Nature*, 392, pp.726–730.

Tomaić, V., Pim, D. & Banks, L., 2009. The stability of the human papillomavirus E6 oncoprotein is E6AP dependent. *Virology*, 393, pp.7–10.

Tonikian, R. *et al.*, 2008. specificity map for the PDZ domain family. *PLoS Biology*, 6, p.e239.

Töpffer, S. *et al.*, 2007. Protein tyrosine phosphatase H1 is a target of the E6 oncoprotein of high-risk genital human papillomaviruses. *The Journal of general virology*, 88, pp.2956–2965.

Trentin, J.J., Yabe, Y., & Taylor, G., 1962. The quest for human cancer viruses. *Science*, 137, pp. 835-841.

Tsoumpou, I. *et al.*, 2009. p16(INK4a) immunostaining in cytological and histological specimens from the uterine cervix: a systematic review and meta-analysis. *Cancer Treatment Reviews*, 35, pp.210–220.

Tsubata, C. *et al.*, 2005. PDZ domain-binding motif of human T-cell leukemia virus type 1 Tax oncoprotein is essential for the interleukin 2 independent growth induction of a T-cell line. *Retrovirology*, 2, p.46.

Tsukita, S. *et al.*, 2008. Tight junction-based epithelial microenvironment and cell proliferation. *Oncogene*, 27, pp.6930–6938.

Tsuruga, T. *et al.*, 2007. Loss of Hugl-1 expression associates with lymph node metastasis in endometrial cancer. *Oncology Research*, 16, pp.431–435.

Um, S.-J. *et al.*, 2002. Abrogation of IRF-1 response by high-risk HPV E7 protein in vivo. *Cancer Letters*, 179, pp.205–212.

Umeda, K. *et al.*, 2006. ZO-1 and ZO-2 independently determine where claudins are polymerized in tight-junction strand formation. *Cell*, 126, pp.741–754.

Ungefroren, H. *et al.*, 2001. FAP-1 in pancreatic cancer cells: functional and mechanistic studies on its inhibitory role in CD95-mediated apoptosis. *Journal of Cell Science*, 114, pp.2735–2746.

Valiente, M. *et al.*, 2005. Binding of PTEN to specific PDZ domains contributes to PTEN protein stability and phosphorylation by microtubule-associated serine/threonine kinases. *The Journal of Biological Chemistry*, 280, pp.28936–28943.

Valle, G.F. & Banks, L., 1995. The human papillomavirus (HPV)-6 and HPV-16 E5 proteins co-operate with HPV-16 E7 in the transformation of primary rodent cells. *The Journal of general virology*, 76 (Pt 5), pp.1239–1245.

Varelas, X. *et al.*, 2010. The Crumbs complex couples cell density sensing to Hippo-dependent control of the TGF-β-SMAD pathway. *Developmental Cell*, 19, pp.831–844.

Vermeer, P.D. *et al.*, 2003. Segregation of receptor and ligand regulates activation of epithelial growth factor receptor. *Nature*, 422, pp.322–326.

Vetrano, S. *et al.*, 2008. Unique role of junctional adhesion molecule-a in maintaining mucosal homeostasis in inflammatory bowel disease. *Gastroenterology*, 135, pp.173–84.

Vinokurova, S. *et al.*, 2008. Type-dependent integration frequency of human papillomavirus genomes in cervical lesions. *Cancer Res*, 68, pp.307–313.

Wang, H.G., Draetta, G. & Moran, E., 1991. E1A induces phosphorylation of the retinoblastoma protein independently of direct physical association between the E1A and retinoblastoma products. *Molecular and Cellular Biology*, 11, pp.4253–4265.

Wang, Q. *et al.*, 2004. Functional Analysis of the Human Papillomavirus Type 16 E1∧E4 Protein Provides a Mechanism for In Vivo and In Vitro Keratin Filament Reorganization. *Journal of Virology*, 78, pp.821–833.

Wang, Z. *et al.*, 2004. Mutational analysis of the tyrosine phosphatome in colorectal cancers. *Science*, 304, pp.1164–1166.

Watanabe, S., Kanda, T. & Yoshiike, K., 1989. Human papillomavirus type 16 transformation of primary human embryonic fibroblasts requires expression of open reading frames E6 and E7. *Journal of Virology*, 63, pp.965–969.

Watson, R.A. *et al.*, 2002. Changes in expression of the human homologue of the Drosophila discs large tumour suppressor protein in high-grade premalignant cervical neoplasias. *Carcinogenesis*, 23, pp.1791–1796.

Watson, R.A. *et al.*, 2003. Activity of the human papillomavirus E6 PDZ-binding motif correlates with an enhanced morphological transformation of immortalized human keratinocytes. *J Cell Sci*, 116, pp.4925–4934.

Weiss, R.S. & Javier, R.T., 1997. A carboxy-terminal region required by the adenovirus type 9 E4 ORF1 oncoprotein for transformation mediates direct binding to cellular polypeptides. *Journal of Virology*, 71, pp.7873–7880.

Welters, H.J. *et al.*, 2008. The protein tyrosine phosphatase-BL, modulates pancreatic beta-cell proliferation by interaction with the Wnt signalling pathway. *The Journal of endocrinology*, 197, pp.543–552.

Weng, Q.P. *et al.*, 1998. Regulation of the p70 S6 kinase by phosphorylation in vivo. Analysis using site-specific anti-phosphopeptide antibodies. *J Biol Chem*, 273, pp.16621–9.

Werme, K., Wigerius, M. & Johansson, M., 2008. Tick-borne encephalitis virus NS5 associates with membrane protein scribble and impairs interferon-stimulated JAK-STAT signalling. *Cellular Microbiology*, 10, pp.696–712.

Werness, B.A., Levine, A.J. & Howley, P.M., 1990. Association of human papillomavirus types 16 and 18 E6 proteins with p53. *Science*, 248, pp.76–79.

Westbrook, T.F. *et al.*, 2002. E7 abolishes raf-induced arrest via mislocalization of p21(Cip1). *Molecular and Cellular Biology*, 22, pp.7041–7052.

Whyte, P. *et al.*, 1988. Association between an oncogene and an anti-oncogene: the adenovirus E1A proteins bind to the retinoblastoma gene product. *Nature*, 334, pp.124–129.

Wieckowski, E. *et al.*, 2007. FAP-1-mediated activation of NF-kappaB induces resistance of head and neck cancer to Fas-induced apoptosis. *Journal of Cellular Biochemistry*, 100, pp.16–28.

Williams, M.R. *et al.*, 2000. The role of 3-phosphoinositide-dependent protein kinase 1 in activating AGC kinases defined in embryonic stem cells. *Current Biology*, 10, pp.439–448.

Williams, J.R., Little, J.B. & Shipley, W.U., 1974. Association of mammalian cell death with a specific endonucleolytic degradation of DNA. *Nature*, 252, pp.754-755.

Wilson, R., Fehrmann, F. & Laimins, L.A., 2005. Role of the E1∧E4 Protein in the Differentiation-Dependent Life Cycle of Human Papillomavirus Type 31. *Journal of Virology*, 79, pp.6732–6740.

Woodman, C.B.J. *et al.*, 2003. Mechanisms of disease Human papillomavirus type 18 and rapidly progressing cervical intraepithelial neoplasia. *The Lancet*, 361, pp.40–3.

Wu, M., Pastor-Pareja, J.C. & Xu, T., 2010. Interaction between Ras(V12) and scribbled clones induces tumour growth and invasion. *Nature*, 463, pp.545–548.

Wyllie, A.H., 1980. Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. *Nature*, 284, pp.555–556.

Xue, B. *et al.*, 2012. Loss of Par3 promotes breast cancer metastasis by compromising cell-cell cohesion. *Nat Cell Biol*, 15, pp.1–14.

Y, H. *et al.*, 2010. Par3 controls spindle pole orientation in epithelial cells by apkc-mediated phosphorylation of Pins at the apical cortex. *Molecular Biology of the Cell*, 21.

Yamanaka, T. *et al.*, 2003. Mammalian Lgl forms a protein complex with PAR-6 and aPKC independently of PAR-3 to regulate epithelial cell polarity. *Current Biology*, 13, pp.734–743.

Yamanaka, T. *et al.*, 2001. PAR-6 regulates aPKC activity in a novel way and mediates cell-cell contact-induced formation of the epithelial junctional complex. *Genes to cells devoted to molecular cellular mechanisms*, 6, pp.721–731.

Yao, R. *et al.*, 2001. Identification of a PDZ domain containing Golgi protein, GOPC, as an interaction partner of frizzled. *Biochemical and Biophysical Research Communications*, 286, pp.771–778.

Yap, A.S., Niessen, C.M. & Gumbiner, B.M., 1998. The Juxtamembrane Region of the Cadherin Cytoplasmic Tail Supports Lateral Clustering, Adhesive Strengthening, and Interaction with p120ctn. *The Journal of Cell Biology*, 141, pp.779–789.

Yoshinouchi, M. *et al.*, 2003. In vitro and in vivo growth suppression of human papillomavirus 16-positive cervical cancer cells by E6 siRNA. *Molecular therapy the journal of the American Society of Gene Therapy*, 8, pp.762–768.

You, J. *et al.*, 2004. Interaction of the bovine papillomavirus E2 protein with Brd4 tethers the viral DNA to host mitotic chromosomes. *Cell*, 117, pp.349–360.

Yu, J.-H. *et al.*, 2007. Mitogen-Activated Protein Kinases Activate the Nuclear Localization Sequence of Human Papillomavirus Type 11 E1 DNA Helicase To Promote Efficient Nuclear Import. *Journal of Virology*, 81, pp.5066–5078.

Zerfass, K. *et al.*, 1995. Cell cycle-dependent disruption of E2F-p107 complexes by human papillomavirus type 16 E7. *Journal of General Virology*, 76 (Pt 7), pp.1815–1820.

Zerfass, K. *et al.*, 1995. Sequential activation of cyclin E and cyclin A gene expression by human papillomavirus type 16 E7 through sequences necessary for transformation. *Journal of Virology*, 67, pp.3375–3383.

Zerfass-Thome, K. *et al.*, 1996. Inactivation of the cdk inhibitor p27KIP1 by the human papillomavirus type 16 E7 oncoprotein. *Oncogene*, 13, pp.2323–2330.

Zhan, L. *et al.*, 2008. Deregulation of scribble promotes mammary tumorigenesis and reveals a role for cell polarity in carcinoma. *Cell*, 135, pp.865–878.

Zhang, B. *et al.*, 2004. Human papillomavirus type 16 E7 protein increases acetylation of histone H3 in human foreskin keratinocytes. *Virology*, 329, pp.189 – 198.

Zhang, B., Chen, W. & Roman, A., 2006. The E7 proteins of low- and high-risk human papillomaviruses share the ability to target the pRB family member p130 for degradation. *Proceedings of the National Academy of Sciences of the United States of America*, 103, pp.437–442.

Zhang, Y. *et al.*, 2007. Structures of a Human Papillomavirus (HPV) E6 Polypeptide Bound to MAGUK Proteins: Mechanisms of Targeting Tumor Suppressors by a High-Risk HPV Oncoprotein. *Journal of Virology*, 81, pp.3618–3626.

Zhi, H.-Y. *et al.*, 2011. PTPH1 cooperates with vitamin D receptor to stimulate breast cancer growth through their mutual stabilization. *Oncogene*, 30, pp.1706–1715.

Zhou, J. *et al.*, 1993. Synthesis and assembly of infectious bovine papillomavirus particles in vitro. *The Journal of general virology*, 74 (Pt 4), pp.763–768.

Zhou, R. *et al.*, 2011. Inhibition of mTOR signaling by oleanolic acid contributes to its anti-tumor activity in osteosarcoma cells. *J Orthop Res*.

zur Hausen, H., *et al.*, 1974. Attempts to detect virus-secific DNA in human tumors. I. Nucleic acid hybridizations with complementary RNA of human wart virus. *International Journal of Cancer*, 13, pp. 650-656.

zur Hausen, H., *et al.*, 1975. Human papilloma viruses and cancer. *Bibliotheca haematologica*, pp. 569-571.

zur Hausen, H., 2002. Papillomaviruses and cancer: from basic studies to clinical application. *Nature Reviews Cancer*, 2, pp.342–350.